Fatty Liver Haemorrhagic Syndrome in Laying Hens: Field and Experimental Investigations

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Abstract

Field and experimental investigations were conducted to study the fatty liver haemorrhagic syndrome (FLHS) in caged laying hens. The main goal of the research was to gain a greater understanding of the aetiology and pathogenesis of this non-infectious disease. The disease is characterized by excessive accumulation of fat in the liver and abdominal cavity, subsequent liver rupture, haemorrhage and sudden death of hens. It has been shown that the balance between hepatic synthesis and secretion of lipids is the key point that regulates hepatic and extrahepatic fat deposition in hens. Liver fat accumulation can be increased by many factors, especially nutrition, housing conditions, and inflammatory challenges. A description of normal and abnormal lipid metabolism in the hen, and consequences for hen health and disease (including FLHS) is given in the literature review (Chapter 2).

The initial study examined mortality of layer flocks kept in three different housing systems (cage, barn and free range) on the Gatton Campus, University of Queensland (Chapter 4). It was shown that there were no significant differences in mortality rates of hens (6.1%, 6.4% and 5.8%, for cages, barns and free range, respectively) between the housing systems but the causes of death were different. The most common cause of death in hens kept in cages was FLHS with 74% of dead hens dying from the condition. In contrast, FLHS only accounted for 0 to 5% of hen mortality in the other systems. Post-mortem of dead hens and body weight monitoring of flocks throughout the laying cycle were recommended as tools to predict FLHS.

An epidemiological survey (Chapter 5) of caged flocks in South-Eastern Queensland was undertaken to explore the prevalence of FLHS in commercial layer flocks. From necropsies of dead birds from 7 flocks of different ages, it was found that approximately 40% of hens died due to FLHS. This result indicated that FLHS continues to be the major cause of mortality in commercial caged laying hens kept in either controlled environment sheds or naturally ventilated sheds. As part of the epidemiological study, a questionnaire was circulated among farms and the responses revealed that there is a general lack of knowledge of FLHS in the industry.

The study of the pathogenesis of FLHS is very difficult as it occurs sporadically and over an extended period of time in the field. In the studies in Chapter 6, an oestradiol (E\textsubscript{2}) hen model was used to study the condition. The administration of exogenous E\textsubscript{2} reproduced the disease in 30 wk old laying hens, and was associated with significant changes in E\textsubscript{2} plasma levels and metabolic...
profiles, increased liver weights, and macroscopic (fat depositions, haemorrhages and haematomas) and microscopic alterations. Hens exposed to E$_2$ and fed *ad libitum* diet experienced severe FLHS and had a higher incidence of FLHS than hens that had their feed intake restricted by 10%. One interesting observation from this study (not reported from other investigators) was the alteration of total leukocyte numbers and plasma fibrinogen concentrations after E$_2$ exposure, suggesting that inflammation (as a part of the acute phase response) contributed to the development of FLHS. Further investigations were conducted using E$_2$ hens injected with lipopolysaccharide (LPS) to simulate an immunological challenge in Chapter 7. This challenge increased the incidence of FLHS. Gene expression levels of important inflammatory cytokines (IL-1$\beta$, IL-6, and IL-18 involved in the generation of systemic and local responses to infection and injury) were evaluated. The mRNA expression of both IL-1$\beta$, IL-6 was greatly up-regulated in E$_2$ and LPS treated hens, with IL-6 giving the greatest increase in the acute phase response (3 h post-treatments), while IL-1$\beta$ gave the greatest response in a later stages (at 24 h) of the response. It was confirmed that the stimulation of fibrinogen synthesis during acute-phase response was mediated by leukocytes and cytokines, and the IL-6 had a prominent role.

Studies conducted in this thesis indicate that FLHS is a significant disease of caged layer hens and impacts on hen health and welfare. The finding of the role of inflammation during elevated circulating levels of oestradiol in inducing FLHS is a useful step in understanding the pathogenesis of this condition. Further studies of these factors and the pathogenesis of FLHS are required. Finally, it will not be possible to develop strategies to reduce the incidence of FLHS until the factors that predispose birds to the condition are fully understood.
**Declaration by author**

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None.
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Keywords

Fatty liver haemorrhagic syndrome, laying hen, oestradiol, eggs, cage housing, body weight, mortality, hepatic inflammation, leukocytes, fibrinogen

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FoR code: 0707, Veterinary Science, 50%
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CHOL</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>E₂</td>
<td>Oestradiol (β2 oestradiol)</td>
</tr>
<tr>
<td>FLHS</td>
<td>Fatty Liver Haemorrhagic Syndrome</td>
</tr>
<tr>
<td>GLU</td>
<td>Glucose</td>
</tr>
<tr>
<td>GGT:</td>
<td>Gamma Glutamyl Transferase</td>
</tr>
<tr>
<td>HCT</td>
<td>Haematocrit</td>
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<tr>
<td>HDP</td>
<td>Hen Day Production</td>
</tr>
<tr>
<td>HGB</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cells</td>
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<tr>
<td>RIA</td>
<td>Radio-immunoassay</td>
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<tr>
<td>TP</td>
<td>Total Protein</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
</tr>
<tr>
<td>wk/wks:</td>
<td>Week/weeks</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
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CHAPTER 1
INTRODUCTION AND OBJECTIVES OF THE STUDY

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1.1 Preface

Fatty liver haemorrhagic syndrome (FLHS) is a non-infectious (metabolic) disease of laying hens characterized by excessive accumulation of fat in the liver and abdominal cavity, liver rupture and haemorrhage, and sudden death (Crespo and Shivaprasad, 2003). It is a major cause of mortality in caged commercial laying hens, resulting in great economic losses to the poultry industry, not only from bird mortality but also from decreased egg production in affected flocks (Squires and Leeson, 1988). Birds affected by FLHS are difficult to distinguish from healthy birds; however decreased egg production, increased body weights (BW) and flock mortality can be “signals” of the presence of this metabolic condition in a layer flock (Julian, 2005; Leeson, 2007).

First described by Couch (1956) in heavy breeds of laying hens during hot weather conditions in the USA, this syndrome created much interest. There were a number of studies conducted by researchers between 1950s and 80s (Akiba et al., 1983; Balnave, 1971; Butler, 1975; Haghighi-Rad and Polin, 1982b; Harms et al., 1982; Harms et al., 1977; Ivy and Nesheim, 1973; Pearson et al., 1978a; Pearson and Butler, 1978b; Pearson et al., 1978b; Polin and Wolford, 1973, 1976, 1977; Ringer and Sheppard, 1963; Wolford and Polin, 1972, 1975), with no success in clarifying the aetiology and pathogenesis or reducing the occurrence of this disorder in commercial flocks. It was an unresolved metabolic disease of laying hens (Hansen and Walzem, 1993). It should be noted that during the 1950s intensive housing system (i.e., battery cages) and high-energy diets were increasingly introduced to the poultry industry worldwide (Couch, 1956), suggesting that these factors may have contributed to the increased prevalence of this disease.
Globally, between 55 to 90% of laying hens are housed in conventional cages (Shields and Duncan, 2008), making FLHS a disease of potential importance in terms of hen welfare and farm profitability. Any progress in preventing this condition will benefit bird health and productivity and lead to increased economic returns for the poultry industry (Cherian, 2007). Since the 1990s, little research has been conducted into the aetiology and pathogenesis of FLHS. Given that over 50% of Australia’s layer flock is housed in cages (AECL, 2011), studies on the FLHS are of current importance.

1.2 The Origin of the Study

1.2.1 FLHS – a disease “rediscovered”

The idea to conduct studies on FLHS in caged hens came from the field. There were several reports from egg producers about increased mortalities in their flocks. The author subsequently undertook random necropsies and observed that most hens had large amounts of coagulated blood in the abdominal cavity and pale livers and the diagnosis was FLHS. As described below, FLHS had been investigated in Queensland some 30 years previously. However, in the intervening period it had either been forgotten or considered as a normal consequence in hens producing eggs.

1.2.2 An “old” disease in Queensland flocks

There had been prior reports of the occurrence of FLHS in commercial laying flocks in Queensland (Neill et al., 1975) suggesting that Queensland probably presents “ideal” climatic conditions that favour the outbreak of this metabolic disorder in egg producing flocks.

Neill et al. (1975) showed that for several years (1970 to 1975) there were a number of outbreaks of FLHS in laying hens in South Eastern Queensland. They pointed out that the sporadic nature of this disease, and the lack of established diagnostic tests contributed to the lack of systematic studies on FLHS. In an attempt to explain the pathological alterations of the liver during FLHS, Neill et al. (1975) used histopathology to determine if liver reticulolysis was specific for FLHS. This was of some significance because previous investigators (Butler, 1975; Hall, 1974) had shown that lysed reticulin was unique among fatty liver syndrome in birds, but in some cases this was associated with a toxicosis (e.g. feeding rapeseed). Nevertheless, in their study, Neill et al. (1975) confirmed that the reticulosis was a definitive lesion of FLHS in Queensland, where the outbreaks were
considered not to be associated with any known toxic principle. It is known that reticulosis produces a structural weakness of the liver which will rupture when under stress (nutritional/toxic, infectious, or environmental) (Neill et al., 1977). However, any correlation between liver fat content and degree of lysis of the reticulin was not found, although the presence of heavy fat infiltration in a structurally weakened liver was thought to have been the reason for massive haemorrhage and death of hens (Neill et al., 1975).

An epidemiological study published in the same year by Grimes (1975), examined two Queensland commercial caged flocks with hens of different strains over a period of eight months. This study used necropsies and laboratory examinations of dead or euthanised sick birds to obtain information on the causes of mortality. Results of this survey revealed a high incidence of FLHS in both flocks (approx. 4% of the flocks) and recognized that affected hens were obese, but still producing eggs at the time of death.

The studies conducted by both Neill et al. (1975) and (Grimes, 1975) were conducted in the 1970s and there is a lack of information on the current situation of FLHS in caged birds in Queensland and in Australia.

1.2.3 Past research and the current industry

Most previous investigators have reported that FLHS is a condition with significantly higher incidence in caged hens than in hens kept in other systems (Butler, 1976; Couch, 1956; Meijering, 1979; Peckham, 1984; Shini et al., 2006; Squires and Leeson, 1988; Ugochukwu, 1983; Weitzenburger et al., 2005). However, a few studies have suggested that FLHS occurs equally in both caged and floor birds (Couch, 1956; Crespo and Shivaprasad, 2003; Harms et al., 1972; Trott et al., 2013). Moreover, past research found increased mortality due to FLHS at temperature extremes (Couch, 1956; Greuel and Hartfiel, 1968; Pearson and Butler, 1978a; Polin and Wolford, 1973; Schexnailder and Griffith, 1973).

Most of the previous studies were conducted many years ago and although they may have general applicability to the current industry, much has changed in the intervening period. The genotype of the modern layer is very different to the bird of 30 years ago as are the diets fed. It is less likely that todays caged birds will experience temperature extremes as most cages are located in thermo-neutral temperatures. Interestingly, birds in controlled environment sheds have lower energy requirements for maintaining body temperature. The unused energy might be the factor that
contributes to a higher positive energy balance and the likely occurrence of FLHS. All these factors indicate that studies of FLHS must examine modern housing systems and current management practices.

1.3 Aims and Objectives of the Study

The overarching hypothesis of this thesis is that “FLHS is a major cause of mortality in Queensland laying flocks”. Following the initial study which demonstrated that FLHS was the major cause of mortality in caged layer flocks but not in other housing systems, further research questions were formulated and experiments designed. These were to:

- Determine the occurrence of FLHS in commercial caged layer flocks;
- Identify environmental factors influencing the incidence of the disease;
- Identify management factors influencing the incidence of the disease.

While conducting these studies it became apparent that to unravel the aetiology and pathogenesis of this disease, a model was needed that would allow the disease to be induced routinely. This became the object of the later studies reported in this thesis.

Ultimately, it was hoped that the work in this thesis would assist the poultry industry to improve the health, welfare and profitability of commercial laying hens.
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2.1 Scope of Review

Metabolic disorders have been recognised for 50 years, yet most still affect the poultry industry (Leeson, 2007).

Metabolic disorders in poultry are those conditions associated with increased metabolism, rapid growth rate or high egg production, that result in the failure of a body system due to an increased work-load on that organ or system (Julian, 2005). Fatty liver haemorrhagic syndrome is classified in that group of metabolic disorders that “result from the failure or deficiency in the production, synthesis or transport of an enzyme, hormone or secretory mechanism” (Julian, 2005).

Fatty liver haemorrhagic syndrome is a disorder of current concern and a major cause of mortality in high producing commercial laying flocks (Leeson, 2007). Although, the liver is the main site of fat synthesis in the chicken, in laying hens fatty liver (FL) is normal (Butler, 1975, 1976). Under the influence of various factors, excessive fat can accumulate in the liver, and the liver capsule can rupture so that haemorrhage occurs and causes death of the bird, and this condition is diagnosed as FLHS. The exact cause of FLHS is still unknown (Julian, 2005; Leeson, 2007; Squires and Leeson, 1988) and the pathophysiological background of this complex metabolic condition is still to be outlined.

For the purpose of this review a broad description of lipogenesis and lipid storage in the chicken is presented, to increase the understanding and help distinguishing the normal pathways of lipid metabolism from “non-normal” (i.e. “disordered”) pathways. Chicken liver structure and function are introduced to provide a logical foundation for the understanding of physiological and pathophysiological events that can occur under the influence of various factors (nutritional and non-nutritional). This review recognises the involvement of liver and non-liver components in the pathogenesis of FLHS. A series of earlier studies are presented here, in particular those focusing on the diagnosis and control of FLHS in laying flocks. Finally, important concluding remarks are given to support studies that follow in the next chapters of this thesis.
2.2 Metabolism of lipids and lipid disorders in laying hens

2.2.1 Introduction

Lipids (i.e. fats) are water-insoluble hydrocarbon derivatives that serve as structural components of membranes, energy-rich fuel stores, pigments, and intracellular signals (Nelson and Cox, 2008). Fats and oils are the principal forms of stored energy in many organisms. Phospholipids and sterols present the major structural components of biological membranes. Other lipids are present in relatively small amounts, but play crucial roles as electron carriers (in mitochondrial membranes, electron transport system), enzyme cofactors (fat-soluble vitamins or their metabolic products), light-absorbing pigments, hydrophobic anchors for proteins, “chaperones” to help membrane proteins fold, hormones, intracellular messengers, emulsifying agents in the digestive tract (Gurr et al., 2002). Last but not least, lipids (i.e. adipose tissue) have insulating properties, important in thermoregulation and protection of internal organs.

Like a mammal, the chicken utilises lipids as its main energy source (Butler, 1975). Dietary lipids are the only dietary component that is deposited intact into tissues with little or no modification (Klasing, 1998). Consequently, the fatty acid composition of the diet primarily determines the fatty acid composition of fat stores in birds, although some conversion of dietary fatty acids occurs creating some differences between fatty acid composition of diet and body fat (Klasing, 1998). Unlike in mammals, lipogenesis in birds is very limited in the adipose tissue and the production of lipids is greater in the hepatic tissue (Hermier, 1997). In laying hens, the liver plays a dominant role in the synthesis and metabolism of lipids. Lipids metabolised in the liver are derived from three main sources: feed fat, depot fat, and from de novo fatty acid synthesis (from feed carbohydrates). Briefly, the digestion and absorption of dietary lipids occurs in small intestine, lipogenesis occurs mainly in the liver, and the transport of triglycerides from liver into the adipose tissue or the oviduct is facilitated by various classes of lipoproteins.

2.2.2 Digestion and absorption of lipids

Birds are very efficient animals at absorbing and utilising fat. Chickens are able to utilize fat at more than 50% of dietary dry mass (Place, 1996). Dietary fatty acids are absorbed mainly in the duodenum and jejunum after being emulsified by lipase and bile salt. Digestion of dietary lipids (basically of triglycerides) in the intestine involves their partial hydrolysis and absorption.
Plasma lipid transport in birds is similar to that in mammals, however due to a poorly developed intestinal lymphatic system in birds, dietary fatty acids are secreted directly into the portal blood system as very low density lipoproteins and are termed portomicrons (Bensadoun and Rothfeld, 1972). The size (mean diameter of about 150 nm) and composition (about 90% triglycerides) of portomicrons are very similar to those of mammalian chylomicrons (Griffin et al., 1982), but their mode of transport differs; portomicrons enter the intestinal blood vessels through endothelial intracytoplasmic vesicles, whereas chylomicrons enter the intestinal lymphatics through gaps between endothelial cells (Fraser et al., 1986). Due to entry into the portal blood system, portomicrons pass through the liver before they reach the rest of the circulation (Figure 2.1). Such a feature predisposes birds to fat deposition in the liver (Cherian et al., 2002). Apparently, these particles are too large to go through the sieve plate-like pores in the sinusoidal capillary bed and cannot be also metabolized by the liver (Fraser et al., 1986); eventually they will be deposited in the liver tissue. Such particles are absent from the plasma of unfed birds and are in very low concentration in fed immature birds (Hermier et al., 1996). However, this situation may also reflect the very rapid catabolism of portomicrons in extrahepatic tissues (e.g. adipose and muscle tissue).

Studies have shown that the ovarian follicle of the laying-hen makes little contribution to catabolism of circulating portomicrons, as it specifically excludes lipoproteins of intestinal origin from yolk, most probably because they are too large to pass through the connective tissue matrix of the basal lamina (Griffin and Perry, 1985).
Figure 2.1   Plasma lipid absorption and transport in birds and mammals (Griffin and Hermier, 1988).

NEFA=non-esterified fatty acids; VLDL=very low density lipoproteins; HDL=high density lipoproteins
*Chylomicrons and lymph in mammals;
Picture adapted from: www2.estrellamountain.edu/faculty/farabee/BIOBK/biobookdigest.html
2.2.3 Lipid metabolism in the laying hen

**Lipogenesis**

As mentioned above, in birds lipogenesis (i.e., the conversion of glucose to triglycerides) takes place primarily in the liver and involves a series of linked, enzyme-catalysed reactions including glycolysis, the citric acid cycle and fatty acid synthesis (Richards et al., 2003). Because commercial avian breeds are usually fed lipid-poor diets (less than 10%), the liver plays a key role in lipogenesis, providing lipids destined to be used by all tissues, including the liver itself (Hermier, 1997). Dietary fatty acids in poultry are provided by animal- or vegetable-blend oils that are high in saturated and n-6 fatty acids. However, avian hepatic cells are able to synthesise saturated fatty acids from non-lipid substrates (i.e. *de novo* synthesis) (Klasing, 1998) and to oxidize them to mono- and di-unsaturated fatty acids. The biochemical details of this synthesis are similar to those in mammals (Donaldson, 1990). Birds, however, cannot use stearic acid to synthesise linoleic and linolenic acids therefore they have a dietary requirement for them. Both dietary and endogenously produced fatty acids are metabolised by enzymes within chicken hepatocytes from arachidonic acid, which is further de-saturated by hepatocytes to produce prostaglandins and eicosapentanoic acid, respectively (Klasing, 1998).

It should be stressed that lipogenesis in the chicken liver is high, and particularly active in females producing eggs. In laying hens, hepatic lipogenesis is dramatically enhanced by oestrogens in order to meet the demand for vitellogenesis (Hermier, 1997). *De novo* hepatic fatty acid synthesis in a female hen is essential to meet the lipid requirement of producing an egg every day which contains 6 g of triacylglycerols transported to the oocyte from the liver (Walzem et al., 1999). Fatty acid synthesis depends on the availability of dietary carbohydrate to ultimately provide acetyl-CoA (Figure 2.2). Although, the main products of *de novo* hepatic lipogenesis are triglycerides, the liver is also the major site of cholesterol and phospholipid synthesis. These lipids, along with proteins, are the main components of lipoproteins. In chickens, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoproteins (HDL) are the three classes of lipoprotein particles that are also synthesized and secreted by the liver (Walzem et al., 1994).
Figure 2.2  The lipogenic pathways responsible for the production of triglycerides (Richards et al., 2003).

It starts from glucose, including the export of triglycerides from the liver to other tissues such as adipose tissue and muscle tissue; specific enzymes are shown next to the steps in the reaction that they catalyse.

**Lipid transport and lipoprotein catabolism**

Fatty acids, triglycerides, and other lipids are almost completely insoluble in blood plasma and tissue fluids. Fatty acids bind to fatty acid-binding proteins (within the cells) and albumin (in body fluids) to be solubilised (Nelson and Cox, 2008). Triglycerides are transported in the blood as components of lipoproteins (Figure 2.3). The liver repackages dietary lipids and endogenous synthesised lipids in the hepatocytes to give VLDL, which is secreted into the circulation. The transfer of triglycerides from VLDL into the adipose tissue involves their catabolism by lipoprotein lipase (LPL) (Hermier, 1997). This enzyme catalyses the hydrolysis of triglycerides into free fatty acids and glycerol molecules, which diffuse into the cells. The fatty acids then enter the surrounding tissues and, in the case of the adipose tissue, they are reesterified and stored as triglycerides. The LPL is synthesized in adipocytes as well as in muscle and other cell types, but only the fraction of the enzyme that has been secreted and anchored to the surface of the capillary
wall is functionally active. In mammals, LPL must be activated by an apolipoprotein (apo) of low molecular weight that is secreted with HDL and then transferred to VLDL prior to their hydrolysis (Griffin and Hermier, 1988).

![Lipoprotein structure](image)

**Figure 2.3** Lipoprotein structure (Alvarenga et al., 2011).

The VLDL is the major lipoprotein responsible for the transport of lipids from the hen’s liver to the oocyte and accounts for 60% of the dry yolk mass (Speake et al., 1998). At the onset of lay, VLDL concentrations rise, while those of HDL are approximately halved (Griffin and Hermier, 1988). The specific protein moieties, apo of these lipoprotein particles are also synthesized in the liver (Siuta-Mangano et al., 1982). Synthesis and secretion pathways of chicken HDL are believed to be similar to those of VLDL. However, it is not known why triglycerides are preferentially associated with apo B into VLDL particles, whereas most of the phospholipids and cholesterol are associated with apo A-I in HDL. Vitellogenin is another lipoprotein synthesized in the liver and functions in the transport of lipids from liver to the oocyte and accounts for about 24% of the dry mass of egg yolk (Speake et al., 1998).

In laying hens, the plasma catabolism of VLDL is very limited (Griffin et al., 1982), which allows the transport of lipids to oocytes, where VLDL are endocytosed, rather than to other tissues. Indeed, laying hen apo VLDL contain large amounts of an apo that is synthesized only under the influence of oestrogen (Nikolay et al., 2013). The intensive synthesis of yolk lipoproteins by the livers occurs faster than their mobilisation from the hepatocytes resulting in a transient increase in liver size and lipid content. Additionally, the rate of clearance of VLDL by the ovarian follicles is not as rapid as
hepatic release, therefore circulating triglycerides increase from 2-10 fold during egg production (Klasing, 1998).

**Lipid Storage**

In chickens, lipids and especially triglycerides are stored in hepatocytes, adipocytes, and growing oocytes (Alvarenga et al., 2011). Triglyceride storage in these compartments depends on the availability of a plasma lipid substrate originating from either the diet or lipogenesis in the liver. Intestinal and hepatic lipids are assembled and secreted as lipoprotein particles. Lipid storage in the oocytes is associated with vitellogenesis (Walzem et al., 1994), while excessive fat accumulation in the liver causes hepatic steatosis and FLHS, which are of much concern. In broilers, excessive accumulation of lipids in the adipose tissue is also a major concern, because most fat depots are lost during meat processing incurring losses for meat producers (Alvarenga et al., 2011).

**Endocrine function of adipose tissue and its implications for metabolism and inflammation**

Adipose tissue (or fat) is a form of loose connective tissue composed of adipocytes. Birds have the ability to store large quantities of excess triglycerides (energy) in the liver, adipose tissue (predominately abdominal fat) and in the yolk of developing oocytes. In mammals, there are two major types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT) with different structural and physiological roles (i.e. endocrine, and energy-storage and thermogenesis, respectively) (Cypess and Kahn, 2010). Brown adipose tissue has never been described in birds or other non-mammalian vertebrates (Mezentseva et al., 2008).

A more modern view would categorize WAT as a complex, essential, and highly active metabolic and endocrine organ (Kershaw and Flier, 2004; Ottaviani et al., 2011). Besides adipocytes, adipose tissue contains connective tissue matrix, nerve tissue, stromovascular cells, and immune cells. Together these components function as an integrated unit. Recent research shows that WAT plays a dynamic role in numerous physiological processes (Figure 2.4) through its secretory and endocrine functions (Coelho et al., 2013; Krug and Ehrhart-Bornstein, 2005). Although storage and release of lipids are major functions of adipocytes, they also use specific lipid and protein molecules for intracellular signalling and communicate with essentially every organ system in the body (Kershaw and Flier, 2004). These include leptin, other cytokines, adiponectin, complement components, plasminogen activator inhibitor-1, proteins of the renin-angiotensin system, and resistin (Coelho et al., 2013; Scherer, 2006).
Figure 2.4  White adipose tissue (WAT) as an endocrine organ and its involvement in physiological and metabolic processes of the body (Coelho et al., 2013).

The most significant physiological functions of WAT (a) and some of the factors secreted by WAT (b).
Leptin was one of the first peptide hormone discovered to be primarily secreted by mature adipocytes, and it has various biological activities, including effects on appetite, food intake and body weight regulation, fertility, reproduction and haematopoiesis (Chan et al., 2006). In addition, adipose tissue secretes a variety of peptides, cytokines and complement factors, which act in an autocrine and paracrine manner to regulate adipocyte metabolism and growth, as well as endocrine signals to regulate energy homeostasis. Adipose tissue is an important site for oestrogen biosynthesis and steroid hormone storage. Adipose tissue is also a major site for metabolism of sex steroids and glucocorticoid steroids (Mohamed-Ali et al., 1998). Alteration of WAT function with subsequent dysfunctional expression and secretion of adipokines plays a key role in the pathogenesis of obesity, diabetes, and other metabolic diseases (Armani et al., 2010).

Avian WAT differ from those of mammals since it has only a limited capacity for lipogenesis. Avian white adipocytes depend on the capture of circulating lipids synthesized in the liver or released by digestion in the gut (Stettenheim, 2000). Moreover, growth of the abdominal WAT in chickens is from a combination of adipose cell hyperplasia (increase in adipocyte number) and hypertrophy (increase in adipocyte volume) up to about 12 to 14 weeks, then it continues mainly by hypertrophy (Ji et al., 2012). The endocrine role of avian WAT remains enigmatic as many of the classical hormones found in mammalian adipose tissue have not been found in avians (Ramachandran et al., 2013).

### 2.2.4 Regulation of lipid metabolism in the laying hen

In animals and humans, appetitive drive and food intake are affected by signals from inside the body and the environment and are under hormonal control together with sympathetic and parasympathetic nervous system inputs (Berthoud, 2008). Environmental signals affecting food intake interact almost exclusively with corticolimbic brain areas (especially with hypothalamic and brainstem structures involved in the control of food intake and energy balance) and are modulated by metabolic signals (Berthoud, 2012). In this way, metabolic pathways and metabolites produced by them are integrated into the regulation of feed intake and energy metabolism. Regulation of fatty acid metabolism depends on energy status and changes in various hormones such as insulin, glucagon and catecholamines (Berthoud, 2012).

Relatively little is known about regulation of adipose tissue deposition and metabolism in chickens (Ji et al., 2012). Most studies have focused on broilers, and have shown that adipose tissue metabolism in the chicken is regulated by energy status and, to a lesser extent by insulin. Unlike in mammals, insulin has minimal effect on glucose uptake in chicken adipose tissue (Tokushima et al.,
2005). Insulin does, however, stimulate uptake of acetate, which is the preferred substrate for de novo lipogenesis in chicken adipocytes, although the magnitude of the effect is relatively modest (Montes et al., 1981). Insulin also does not inhibit lipolysis in chicken adipose tissue; glucagon is the primary lipolytic hormone (Scanes, 2009).

Very little is also known about the regulation of lipoprotein synthesis and secretion in avian liver, at least in growing birds. Tarlow et al. (1977) used chicken hepatocytes and demonstrated that insulin enhances both de novo lipogenesis and VLDL synthesis, whereas thyroxine and glucagon have opposite effects. However, lipid secretion as lipoproteins may be not tightly coordinated with lipid synthesis. There is a lack of information on the hormonal regulation of LPL in birds. In fed mammals, LPL activity is enhanced in adipose tissue but is low in muscle, which results in fat storage. The opposite is seen in unfed animals. In birds, LPL regulation in adipose tissue seems to be less sensitive to the nutritional state (Hermier, 1997). Very high concentrations of insulin, fat feeding and refeeding stimulate LPL activity in chicken adipose tissue; however it has been shown that differences in LPL activity are not related to differences in rate of body fat deposition (Borron et al., 1979).

2.2.5 Disorders of lipid metabolism in the laying hen

Disorders of lipid metabolism are a group of metabolic diseases that occur in a variety of species including chickens. Disturbance in lipid and lipoprotein metabolism are associated with abnormalities of lipid storage and lipid mobilization. Hyperlipidemias are of clinical relevance not only in humans but also in chickens, since they constitute an important risk factor for the development of hepatic lipidosis and subsequent diseases, such as vascular and heart disease or FLHS, respectively. Specific lipids accumulate due to the lack of corresponding degradative enzymes (Schulze et al., 2009). Since enzymes also require vitamins and co-factors to properly function, nutritional deficiencies can also be a factor that influences lipid metabolism.

Lipid metabolic disorders affect the hepatocyte’s ability to digest, process, and synthesize essential compounds. Although, there are many factors (i.e. nutritional, genetic, and environmental) that initiate the accumulation of fat in the liver, in many cases the increase in liver lipid synthesis is not accompanied by equivalent increases in lipid outflow, resulting in lipid accumulation. Fatty liver disorder is a reversible condition where large vacuoles of triglyceride fat accumulate in liver cells via the process of steatosis (Brunt, 2010). Fatty liver syndromes in birds (i.e. hepatic steatosis or hepatic lipidosis) have all been used to describe the condition of fatty infiltration of the liver
(Crespo and Shivaprasad, 2003). It occurs in laying hens when the increase in lipogenesis exceeds the capacity of synthesis and secretion of lipoproteins, especially when hens are fed a low protein and high calorie diet. In this case a dramatic enhancement of lipogenesis by oestrogen is responsible for an increase in VLDL secretion (Squires and Leeson, 1988).

Hepatic steatosis is frequently confused with FLHS however the two conditions are quite different (Diaz et al., 1994). Hepatic steatosis causes a drop in egg production but little increase in mortality: FLHS causes increased mortality due to liver haemorrhage and hypovolemic shock. Moreover, hepatic steatosis is caused due to a specific accumulation of triglyceride within the parenchymal cells. The reason why neo-synthesised triglycerides are channeled towards intracytoplasmic storage rather than secretion remains unclear (Hermier, 1997). It is possible that, because the overfed chickens are never deprived of food, hormonal regulation does not allow the liver to secrete the excess of triglycerides, which continue to accumulate (Hermier et al., 1989). The degree of steatosis might vary being an apparent predisposing factor for FLHS (Polin and Wolford, 1977). Excessive hepatic lipid in certain instances leads to the development of fatty liver which is accompanied by liver haemorrhage.

For more details on FLHS see Chapter 2.4.

2.3 A review of the liver and liver diseases in the laying hen

2.3.1 Introduction

As in mammals, in birds the liver is the biochemical factory responsible for most of the synthesis, metabolism, excretion, and detoxification processes, and is involved with many metabolic and homeostatic functions. It plays an important role in digestion and metabolism, regulating the production, storage, and release of carbohydrates, lipids and proteins (Denbow, 2000). The liver produces a variety of proteins, including enzymes, hormones, blood proteins, clotting factors, and immune factors. Because it participates in so many vital functions, the liver in birds is frequently involved in many infectious and non-infectious diseases.

2.3.2 The structure and function of the avian liver

The liver in chickens is the largest internal organ, is firm, and has prominent sharply defined edges. In birds, as in mammals, the liver is a bilobed organ (Figure 2.5). The right lobe is larger, but both are joined cranially at the midline (Lumeij, 1994). Generally, the caudal border of the right lobe
extends to the edge of the sternum. In chickens, bile is transported to the duodenum via two ducts. The right hepatic duct and left hepatic duct combine to form the common hepatoenteric duct which then goes to the distal ascending loop of duodenum (Denbow, 2000). The avian liver has much less connective tissue than the mammalian liver, and it is larger than in mammals when compared to body size (Lumeij, 1994). The liver lobule is formed by parenchymal cells (hepatocytes) and non-parenchymal cells. Hepatocytes occupy almost 80% of the total liver volume and perform numerous liver functions. Non-parenchymal liver cells are localised in the sinusoidal wall and consists of different cells: endothelial cells, immune cells and fat storing cells (Kmiec, 2001). The size and colour of the liver depends on age and body weight. The liver performs numerous physical, physiological and immunologic functions in birds (Figure 2.6). It functions as both an endocrine and exocrine gland (Denbow, 2000).

![Normal liver in the chicken 16 wk of age (a) and 30 wk of age (b).](image)

Figure 2.5   Normal liver in the chicken 16 wk of age (a) and 30 wk of age (b).

Bile acids secreted by liver function to emulsify fats and activate pancreatic lipase and amylase, all of each aid in digestion (Lumeij, 1994). Apart from metabolism of fats carbohydrates, proteins, and detoxification of drugs and toxins, the liver synthesises blood clotting proteins and proteins involved in immunity (acute-phase proteins and globulins). The significant involvement of the liver in controlling systemic innate immunity in avians is due to the fact that birds do not possess definite lymph nodes, rather they possess patches of lymphoid tissue, so that the role of the liver is crucial (Lumeij, 1994). Liver disease or damage, including hepatic lipidosis, may make a bird exceptionally susceptible to agents normally dealt with by the liver. In the case of hepatic lipidosis, normal liver cells are gradually being filled with fat, and these damaged cells can no longer function to perform the liver's work efficiently. As liver cells die, they are replaced with scar tissue or fibrous connective tissue (Hoerr, 1996).
Figure 2.6 The metabolic functions of the liver and the flow of products through the portal vein, hepatic artery, inferior vena cava and common bile duct, the location of central veins and portal tracts, as well as the direction of blood flow (Thomson and Knolle, 2010).
2.3.3 Liver diseases in laying hens

Liver diseases (or hepatopathies) occur sporadically in laying hens. According to the aetiology some of the liver disorders (i.e. hepatitis) have been associated with infectious disease (bacteria, chlamydiosis, viruses, helminths and protozoa), non-infectious disease (such as heart disease complex and other metabolic disorders), gastrointestinal problems and toxins (mycotoxins, rapeseed, cotton seed and other toxic substances) and neoplasia (Lumeij, 1994). Many bacteria can cause hepatitis in birds including Borrelia, Escherichia coli, Salmonella typhimurium, Staphylococcus, Campylobacter, Pasteurella (Gerlach, 1994). Clinical signs may indicate liver disease which can be confirmed by liver functional tests and histologic examination of a liver biopsy (Grunkemeyer, 2010). However, the aetiology for many liver diseases cannot be determined. The distinction between primary and secondary hepatic disease is difficult but very important for the treatment (Jaensch, 2000).

2.3.4 Clinical examination and functional tests of the liver

Clinical signs of hepatopathies in birds are variable and can range from mild inappetence and inactivity to acute haemorrhage and death. The liver has a large functional reserve; therefore clinical signs can be observed only if a large proportion of liver tissue is affected (Hochleithner et al., 2006). Hepatic failure is associated with yellow or green faeces, poor feathering or colour changes (dark), diarrhoea and weight loss (Lumeij, 1994). Comparative studies have shown that the regenerative processes of the liver tissue in birds, as in the other vertebrates, are remarkable and are accomplished by regenerative hypertrophy; however, the original shape of the traumatized part is not restored (Sidorova, 1962). The destruction and regeneration of hepatic parenchyma results in fibrosis; therefore hepatic dysfunction can occur after severe injury or repeated significant damages (Hochleithner et al., 2006; Hoerr, 1996). At necropsy, the liver is the first and largest organ seen when the body cavity is opened. Many lesions in the liver might not be specific to the diagnosis, but provide important information about the general disease process. Changes in shape and colour can help to identify the cause of death (Jaensch, 2000).

Microscopically, the unit of the liver is the lobule (Figure 2.7). Each lobule (or portal lobule) is bounded by four to five portal triads (supplied from the portal vein and hepatic artery) and has a central terminal hepatic venule (central vein). From a functional point of view the basic unit of the liver is the acinus (or zones of hepatocytes between central veins) (Malarkey et al., 2005). The acinus model (Figure 2.8) has at the centre the blood supply to liver parenchyma (portal triad),
rather than the venous drainage (central vein) (Godoy et al., 2013). Hepatocytes make up the bulk of the organ (80%). They are arranged in plates that radiate out from each portal triad toward adjacent central veins. The bile canaliculus is formed by grooves on the contact surface of adjacent liver cells. Bile forms in these canaliculi and progressively flows into ductules, interlobular bile ducts and then larger hepatic ducts (Denbow, 2000).

**Figure 2.7** The lobule model shown in a normal microscopic view of laying hen liver. Hepatocytes (H), central vein (CV), hepatic venule (HV), and bile ductile (BD).
Figure 2.8  The acinus model shown in a normal microscopic view of avian liver.

The basic functional unit of the liver, the hepatic acinus with hepatocytes (H), portal/hepatic triad (HT), and central veins (CV).
Endothelial cells of the liver lack a basement membrane and contain numerous fenestrae that permit hepatocytes to have ready access to nutrients and macromolecules in plasma. Endothelial cells are also responsible for endocytosis of molecules and particles, and play a role in lipoprotein metabolism (Hochleithner et al., 2006). Microvacuoles of fat in the hepatocytes of chicks in the first week of life, and in laying hens are normal (Cherian, 2007). It is arguable what can be considered as a normal or excessive (pathologic) amount of fat in the liver of a laying hen (Butler, 1976).

Percutaneous liver biopsy provides important diagnostic information at relatively low risk, but is needed in only a minority of cases of hepatic dysfunction. A small core of liver tissue can be obtained by needle aspiration under local anaesthesia (Lumeij, 2008). This usually provides a reliable reflection of the underlying disorder, although it is only a cytologic sample, and it is usually inadequate for full histological assessment (Jaensch, 2000).

Determination of a panel of plasma enzymes is often referred to as liver function testing. This is a misleading use of this term, because plasma enzyme concentrations primarily reflect the degree of hepatocellular damage and leakage, not hepatic function, and in many cases may indicate damage to, or dysfunction of, other organs or tissues (Jaensch, 2000). Enzyme panels may include any of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), glutamyl dehydrogenase (GLDH) and creatinine kinase (CK) (Lumeij, 2008). The level of serum ALT activity reflects damage to hepatocytes and is considered to be a highly sensitive and fairly specific preclinical and clinical biomarker of hepatopathology (Ozer et al., 2008). Serum AST and CK activity is considered a less specific biomarker of liver function compared to ALT activity. Serum GLDH activity increases with hepatocellular damage.

Liver function tests provide information on clearance and metabolizing ability or synthesizing ability of the liver (Jaensch, 2000). Synthesizing ability can be assessed by bile acids, plasma albumin concentrations or assay of clotting factors (Hochleithner et al., 2006). In conclusion, no single test can assess overall hepatic function, as the liver is a complex organ with interdependent metabolic, excretory and defence functions. A number of laboratory tests are usually combined to detect hepatic abnormalities, assess their severity, follow the course of the disease, and help establish an aetiology and treatment.
2.4 Fatty liver haemorrhagic syndrome in the laying hen

2.4.1 The aetiology of FLHS

Many studies have tried to identify the aetiology of FLHS; however it is still poorly understood. Important factors that have been considered as contributors into the aetiology of the FLHS are discussed below.

**Nutritional factors**

It has been previously recognised that feeding high producing hens (whose exercise is restricted) high-energy diets will result in the development of fatty livers and potentially FLHS (Akiba et al., 1982; Butler, 1976). However, excessive consumption of feed in hens does not always result in a positive energy balance and increased fat deposition in the liver. The fact that FLHS can be experimentally induced through force-feeding and or oestrogen administration indicates that the condition might be caused by an excess of energy rather than being specific to an excess of any nutrient such as fat or carbohydrate (Squires and Leeson, 1988). Butler (1975) suggested that excess fat in the liver arises mainly from increased lipogenesis rather than from dietary lipids. Other studies have indicated that high energy diets, especially maize or wheat diets produce higher incidences of FLHS (Haghighi-Rad and Polin, 1982b; Pearson et al., 1978a; Polin and Wolford, 1976).

**Other dietary factors**

Dietary factors other than excessive caloric intake, such as toxins and rapeseed products have been shown to stimulate lipogenesis (Pearson et al., 1978b). There is evidence that mycotoxins (aflatoxin in particular) which may contaminate cereals induce liver lipid accumulation (Bryden et al., 1979). Rapeseed meal in the diet increases the incidence of FLHS because erucic acid or other toxic metabolites affect the strength of the connective tissue in the liver (Bhatnagar et al., 1980; Martland et al., 1984). Hens overfed a purified diet had a 33% incidence of FLHS (Walzem et al., 1993). Branton et al. (1995) observed a high incidence of FLHS in hens that consumed diets containing chelated minerals.
Hormonal factors

It has been shown that high oestrogen levels result in increased feed intake and subsequently in a positive energy balance. High producing birds within a flock are most often affected by FLHS (Scheele, 1997), most probably due to a relationship between energy metabolism and hormone levels during egg production. Oestrogens influence lipid synthesis which is required for the yolk (Walzem et al., 1999). Polin and Wolford (1977) indicated that the haemorrhage score in liver was markedly increased when excess energy intake was combined with exogenous oestrogen treatment. The possibility of a hormonal imbalance has been suggested by the observation of greatly elevated serum calcium and cholesterol in chickens from flocks with FLHS (Branton et al., 1995; Harms and Simpson, 1979; Miles et al., 1983).

Genetic factors

There is little proof of the genetic strain influencing the occurrence of FLHS in a layer flock. Experimental studies have indicated that Rhode Island Red hens are more sensitive to experimental induction of FLHS than White Leghorn hens (Stake et al., 1981). It has also been suggested that some strains of laying hens are naturally more susceptible to FLHS (heavy and higher producing breeder hens) (Couch, 1956). Moreover, a strain of single comb white leghorn laying hens (UCD-003) has been shown to be highly susceptible to FLHS (Abplanalp and Napolitano, 1987). Differences between genetically lean and fat chickens are by nature polygenic, and it is obvious that tendency to leanness or fatness in poultry relies on fundamental metabolic differences in the partitioning of nutrients, in which hormones are likely to play a major role (Squires and Leeson, 1988).

Housing and environmental temperature

A high prevalence of FLHS in caged birds is thought to be related to the lack of exercise combined with a high feed intake in this housing system (Jensen et al., 1976a; Shini et al., 2006; Weitzenburger et al., 2005). There are other factors that have been considered to influence the prevalence of FLHS in caged birds, especially temperature. Most investigators have shown that increased lipogenesis occurs partly due to an excessive intake of carbohydrate brought about by hot weather (Couch, 1956; Pearson and Butler, 1978a). Jensen et al. (1976b) observed more FLHS in warmer vs. cooler regions of Georgia, whereas it is known that feed intake is attenuated at high temperature, and increased in cold temperatures. It should be noted that exposure to cold or heat
(stress) stimulates lipolysis in the domestic fowl (Annison, 1983). There is evidence that the injection of adrenocorticotropic hormone (ACTH) also produces a similar response (Jaussi et al., 1962).

**Stress**

As mentioned above, stressful conditions (physical, chemical and biological stresses) activate a coordinated neuroendocrine response associated with increased levels of stress hormones, including catcholamines and ACTH; ACTH is controlled mainly by corticotropin release hormone (CRH). When the 4-wk-old and 8-wk-old chickens were treated with one administration of ACTH (Breitenbach, 1962), the secretion of corticosteroids increased. The glucocorticoid levels were increased from 17 to 22 μg % 1h after ACTH injection. The studies of Hillman et al. (1985) confirmed that experimental stress affected the secretion of corticosterone and its concentrations in the plasma.

When administered with corticosterone, the hepatic lipids increased (Bartov, 1982). Clark and Das (1974) reported that the hepatic tissue of heat stressed chickens had a fatty, pale appearance, with small areas of haemorrhage similar to the appearance the FLHS. However, while the birds were kept at 21 to 38ºC, the liver weight did not change. There are other investigators that have demonstrated that the stress associated with high temperature and humidity of the environment affect the liver of hens and predisposes them to FLHS (Akiba et al., 1983; Ayo et al., 2011; Pearson and Butler, 1978a)

**Bacterial endotoxins**

The possibility of implication of bacterial endotoxins in the pathogenesis of fatty liver haemorrhagic syndrome in the laying hen was investigated by Curtis et al. (1980); Pearson et al. (1981), who showed that the haemorrhage was not ameliorated by suppressing enteric bacteria with neomycin or exacerbated by the repeated injection of *Escherichia coli* O111 endotoxin. It was also decided that the steatosis does not impair the ability of the liver to inactivate endotoxins of enteric bacteria and that these toxins were not involved in the pathogenesis of the syndrome. More recent data from humans and rodents indicate involvement of inflammation in the pathogenesis of fatty liver disorders and a contribution of the gut microbiota to the development of non-alcoholic fatty liver disease (NAFLD) (Alisi et al., 2012; Le Roy et al., 2013).
2.4.2 New insights into the aetiopathophysiology of NAFLD in humans

Recent evidence indicates that the integration of metabolic, immune and inflammatory pathways is crucial, and dysfunction may underlie many chronic metabolic diseases, including NAFLD, which is the major cause of abnormal liver function in humans, and often associated with obesity and diabetes (Qureshi and Abrams, 2007; Tilg and Moschen, 2008).

Adipose tissue and the liver constitute an interesting organ pair that is in constant communication with each other via adipokines, lipid factors, and lipoprotein particles. One of the first organs to be affected when adipose tissue becomes dysfunctional and inflamed is the liver (Attie and Scherer, 2009). A great majority of NAFLD cases occur in patients who are obese, and have enhanced dietary fat delivery and physical inactivity. Altered physiology of adipose tissue is seen as central to development of insulin resistance, metabolic syndrome and NAFLD (Attie and Scherer, 2009; Qureshi and Abrams, 2007). Similar metabolic disorders (hepatic lipidosis and FLHS) that occur in caged laying hens are also associated with diet, increased feed intake, high egg production and lack of exercise.

Lipid accumulation in the liver is the early and relevant pathophysiological step of the development of NAFLD, which remains a benign condition in most affected individuals (Kirovski et al., 2010). The proinflammatory state in the liver observed in NAFLD is regulated by an imbalance of various cytokines, transcription factors and adipocytokines (Tilg and Moschen, 2008). A hallmark of NAFLD is that fat accumulation and inflammatory processes are taking place in parallel and, therefore, a better understanding of such pathways is crucial. Another mystery in human studies is why simple steatosis remains a benign condition in most affected individuals, and why transition to an inflammatory condition, such as NASH, takes place.

2.4.3 Clinical and pathological aspects of FLHS in laying hens

First changes in the liver can be observed at the onset of the reproductive period, and are related to the increase in synthesis of lipids and proteins destined for the egg-yolk. However, no clinical signs are detected at this stage. The most profound changes occur at or after the peak of lay (from 35 to 40 wk of age) most probably induced by oestrogen persistence throughout the laying period; at this stage clinical signs like increased BW, reduced egg production, sudden deaths, or hens with pale
combs and becoming lethargic can be recorded in a flock (Grimes et al., 1991; Harms et al., 1972; Julian, 2005; Squires and Leeson, 1988; Walzem et al., 1993).

On post-mortem examination, attention is initially focussed on the extreme accumulation of fat in the abdominal cavity and the visceral regions (Butler, 1976; Scheele, 1997). The liver is enlarged, rounded and very fragile; its colour varies from light brown to yellow (Crespo and Shivaprasad, 2003; Hoerr, 1996). In the liver parenchyme numerous capillary bleedings (haemorrhages), varying in size, can be observed (Couch, 1956; Hoerr, 1996). These haemorrhages may extend to the surface, or can form hematomas under the liver capsule. Mortality occurs when the degeneration of the liver tissue results in a rupture of the liver capsule, causing a fatal internal bleeding.

Microscopic examination of the livers from hens with FLHS may show liver cells containing diffuse small fat droplets. More often, liver cells contain large droplets or one large fat vacuole that almost completely covers the cytoplasm and causes a change of cell colour and a displacement and deformation of the nucleus (Cherian and Goeger, 2004; Hoerr, 1996; Jaensch, 2000; Walzem et al., 1993).

### 2.4.4 Experimental approaches to studying FLHS in a chicken model

There have been a number of attempts to reproduce FLHS in chickens and study the involvement of oestrogen and feed intake in the pathogenesis of FLHS. Polin and Wolford (1977) induced FLHS in immature male and female chickens, 11 weeks of age, of broiler and egg-laying breeds by force-feeding birds three times a day for 21 days, amounts of feed equal to 125% and 150% of ad libitum intake, and produced a gradient response in hepatic steatosis (as measured by percentage of fat in the liver, and the ratio of fat to the fat-free dry weight of the liver). However, they were not able to reproduce FLHS. Intramuscular injections of β-estradiol-17-dipropionate (E₂) at 2 mg/kg body weight, three times weekly for 21 days, produced a gradient response in haemorrhagic score, and an increase in ad libitum feed intake. Polin and Wolford (1977) did not find any significant difference between sex or breed in the score values used to evaluate FLHS, but females of both breeds accumulated significantly more fat in the liver than males. Their studies showed that oestrogen is implicated in the production of FLHS, along with the necessity for the chicken to be in a positive energy balance induced FLHS to occur.

Stake et al. (1981) used i.m. administration of E₂ every 4 or 5 days (5.0 or 7.5 mg E₂/kg body weight) to induce FLHS in both Rhode Island Red (RIR), and White Leghorn (WL) hens. RIR hens
exhibited ataxia and opisthotonus, and 30% died from hepatic haemorrhage within 14 days. No WL birds similarly treated for 32 days died or showed neurologic disorder, thereby indicating a major breed difference in response to exogenous E2.

Other investigators have shown that exogenous E2 combined or not with an increase in feed intake or use of high energy diets can induce fatty liver in immature, mature or older birds (Akiba et al., 1983; Balnave, 1971; Chawak et al., 1997; Haghighi-Rad and Polin, 1981, 1982a; Harms et al., 1977; Martland et al., 1984; Pearson and Butler, 1978b; Schumann et al., 2003; Walzem et al., 1993; Yamashiro et al., 1975). It is thought that the induction of FLHS in mature layers may be a better model to study the pathogenesis as FLHS is more often observed in high producing layers.

2.4.5 Monitoring and control of FLHS in laying flocks

Since the first descriptions of the FLHS in 50s, monitoring and preventive strategies have been searched. Various aspects of nutrition have been regarded in terms of diet restrictions and diet manipulations, with little or no success.

There is no evidence about any attempt to conduct regular monitoring of laying flocks for FLHS. Most of the investigators suggest regular monitoring of BW, egg production and mortality rates, as these records may indicate the presence of FLHS in a flock. While mortality may be low, the number of subclinical cases of FLHS could be much higher. Many hens experience excessive fatty liver or liver haemorrhages but not all die from FLHS. Hens that survive from moderate hepatic haemorrhages or haematomas located within the subcapsular membrane of liver continue to live with or without a decline in egg production. Overweight hens are predisposed to FLHS (Couch, 1956), in particular hens with body weights heavier than the breeder’s recommendation for their age and phase of production are more susceptible to FLHS (Meijering, 1979). In the case of natural outbreaks of FLHS, hens that do not die suddenly exhibit pale combs (Grimes et al., 1991; Harms et al., 1972). However, it has been shown that hens with normal comb colour still had liver haemorrhage, indicating that comb appearance alone is not completely indicative of FLHS. No definitive diagnostic criterion has been determined for FLHS in live birds, so the diagnosis is usually made at necropsy (Couch, 1956; Grimes et al., 1991; Meijering, 1979; Squires and Leeson, 1988; Thomson et al., 2003).

If lipogenesis exceeds the capacity of VLDL secretion, triglycerides accumulate in the liver. One way to reduce FLHS could be the control of VLDL production. Previous investigators have
undertaken nutritional trials to reduce lipogenesis (e.g. a partial replacement of dietary energy by protein) and subsequent fattening, which have been shown to be effective, but high-priced (Hermier, 1997). Other attempts have been made to restrict the energy intake (i.e. restricting feed intake). A severe feed restriction (80 % of the intake of the control group) during six weeks resulted in a drop in body weight, weight of the abdominal fat pad, liver weight and mean liver fat content (Polin and Wolford, 1976; Wolford and Murphy, 1972; Wolford and Polin, 1974). Despite the positive effect of restricted feed intake on reducing FLHS, this preventive measure was found less suited, due to difficulties in setting the level of restriction in practical situations. Reduction of the energy content of the feed from 2900 to 2400 kcal ME/kg reduced liver fat content (Wolford and Murphy, 1972). Adding a variety of lipotropic compounds, fatty liver supplements or antioxidants to the diet for their protective effects on the liver has been shown to be effective in controlling FLHS in hens (Diaz et al., 1994; Harms et al., 1982; Spurlock and Savage, 1993; Wolford and Polin, 1975; Yeh et al., 2009).
2.5 Concluding remarks

Although each metabolic disorder has unique predisposing factors, increased productivity, either as egg output or growth, is a common factor (Leeson, 2007).

Due to advances in genetic selection, nutrition, and other management practices, the “modern laying hen” demonstrating improved metabolic and egg production phenotypes might experience failure of a body system because of the increased work-load on an organ or system such as the liver (Julian, 2005). The liver is the major site of synthesis of lipids and the majority of proteins, including enzymes, hormones, blood proteins, clotting factors, and immune factors are synthetised in the hepatocytes. When hepatic lipogenesis exceeds the capacity of VLDL secretion, triglycerides accumulate in the liver, causing steatosis. The balance between synthesis and secretion of VLDL is therefore a key point that may regulate hepatic and extrahepatic fat in poultry (Hermier, 1997).

As described in this review, the laying hen appears to develop fatty liver under conditions of excess energy intake. Additionally, other environmental factors provide a possible influence in terms of alteration of liver function and lipid utilisation, and therefore mediate the accumulation of fat in the liver predisposing laying hens to FLHS. Despite its obvious importance, the aetiology of FLHS is not been clearly defined and the pathogenesis is poorly understood. Moreover, no definitive diagnosis criteria have been determined for live birds.

Previous studies (Polin and Wolford, 1977; Stake et al., 1981) implicate oestrogen as a factor in the production of FLHS along with the necessity for the chicken to be in a positive energy balance creating sufficient hepatic fat for FLHS to occur. These studies used immature chicken as a model to induce FLHS. The induction of FLHS in mature hens may prove to be more useful than younger hens, since FLHS is commonly observed in high producing layers. Recent studies in humans have provided new information regarding communication between adipose tissue and the liver via adipokines and other molecular factors. The liver is the first organ to be affected when adipose tissue becomes dysfunctional and inflamed, initially undergoing a proinflammatory state followed by inflammatory processes. With respect to this in chickens, it will be important to study the pathogenesis of FLHS in a reproduced laying hen model to see if certain molecular factors influence fat accumulation and inflammatory processes in the liver of affected hens. Moreover, it will be intriguing to understand why only some laying hens develop FLHS, while all of them have fatty livers.
CHAPTER 3

GENERAL MATERIALS AND METHODS

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3.1 Birds and bird care

3.1.1 Laying hens and housing conditions

For the first study, hens (Hy-Line Brown layers) were housed in three different housing systems: conventional cages (in a controlled environment shed), free range and barn systems. Detailed information on the housing conditions is given in Chapter 4. Production and health parameters were recorded for 52 weeks. Husbandry procedures were similar for all birds, and the same personnel were responsible for handling birds in all housing systems.
For the second study, the questionnaire was sent out to 20 registered farms in Queensland, and data were collected from birds used and kept in these farms. For the epidemiological study, birds of three farms involved in the monitoring were kept at their respective farms. Samples were collected on site while data on performance were provided by producers (see Chapter 5 for more details).

For experiments 3 and 4 (Chapters, 6 and 7, respectively), Hy-Line Brown laying hens were used. Pullets, 17 wk old birds, were housed in a controlled shed (at the Layer Facility, Poultry Unit at the Gatton Campus, University of Queensland). Hens were housed individually in stainless steel cages and kept in a climate controlled environment. The temperature of the shed ranged from 22ºC to 24ºC, and hens were exposed to a photoperiodic lighting 16 hours of light: 8 hours of dark. Hens were kept in the shed until they were 30 wk of age and then used to reproduce FLHS.

In all experiments, husbandry and care were in accordance with the animal welfare guidelines established at University of Queensland, and with ethical clearance obtained by the University Animal Ethics Committee, approval numbers SAS/871/07, and SAS/842/08.

### 3.1.2 Diets

Commercial layer diets and water were available *ad libitum* in all experiments. Details of the diet used in 3 farms during the epidemiological survey (Chapter 5) appear in Appendix 1, and for all other experiments carried out at Gatton in Appendix 2. For the investigations of FLHS in different housing systems, layers in all systems were fed the same commercial diet (Appendix 2). In all systems, feed and water were available *ad libitum* for consumption.

### 3.2 Collection of samples

#### 3.2.1 Blood

For all experiments blood samples were taken in the morning between 8:00 and 10:00 a.m., immediately after each hen had been removed from its cage. Each bird was appropriately restrained to ensure as little stress as possible on the bird; the entire procedure did not take longer than 45 to 50 s.
For haematological analyses whole blood was used and samples were taken from the wing vein using individual EDTA vacutainers and individual blood tubes. For the oestradiol assay and metabolite profile, blood samples were centrifuged at 1500X g for 10 min and plasma was divided into three aliquots and stored at -20°C until subsequently analysed.

### 3.2.2 Organs

For studies in Chapter 6 and 7, hens were euthanised by cervical dislocation and subjected to post-mortem examination. After dissection, liver, ovary, and oviduct were removed and weighed. The absolute and relative weights (organ weight to body weight ratio) of organs were calculated. Liver samples were used to prepare slides for histology examinations.

### 3.2.3 Eggs

Eggs were collected daily. Egg production was calculated per day as hen-day production (HDP) or number of eggs produced per day and expressed as percentage. The following measures of egg quality were determined: egg weight, shell thickness, shell weight, albumen height (from which Haugh Units were calculated), and yolk colour score (Roche Egg Yolk Colour Fan, Switzerland).

### 3.3 Methods

#### 3.3.1 Oestradiol determination

**Principle of the test**

The procedure follows the basic principle of radioimmunoassay (RIA) where there is competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. The amount of [I-125]-labelled oestradiol bound to the antibody is inversely proportional to the concentration of the oestradiol present in the sample. The separation of free and bound antigen is easily and rapidly achieved by using a double antibody system.

Plasma 17-\(\beta\) oestradiol concentration was determined by RIA kit using coated tube technology (Spectria, Orion Diagnostica Oy, Finland). The RIA was conducted according to the manufacturer’s instructions using duplicate 200 \(\mu\)L samples in assays. Samples were counted on a Gamma Counter.
(Wallac 1470 Wizard Automatic). At 394 and 9,520 pmol/L the inter-assay variations were 5.1 and 8 % respectively, and the intra-assay coefficient of variation was 4.33%. The assay sensitivity ranged between 30-15,000 pmol/L. The antiserum was highly specific for 17-β oestradiol with a relatively low cross reactivity to other naturally occurring steroids in the plasma sample as stated by the manufacturer.

**Assay procedure**

All (samples standards, controls and unknowns were assayed in duplicates).

1. 200 μL of the standards, controls or unknowns were added to appropriate tubes. 300 μL of the 0 pg/mL oestradiol standard was added to Non-Specific Binding (NSB) tubes.
2. 100 μL of oestradiol antiserum was added to all tubes except NSB and total count tubes.
3. All tubes were vortexed, covered and incubated at room temperature (25°C) for 1 hour.
4. 100 μL of oestradiol [I-125] reagent was added to each tube.
5. All tubes were vortexed, covered and incubated at room temperature (25°C) for 2 hours.
6. 1 mL of precipitating reagent was added to all tubes except total count tubes. Tubes were vortexed and allowed to stand at room temperature (25°C) for 15-20 minutes.
7. All tubes were centrifuged (refrigerated), for 15-20 minutes at 1500 x g, except the total count tubes.
8. The content was decanted from all tubes (except total count tubes) by simultaneous inversion with a sponge rack into a radioactive waste receptacle.
9. Tubes were allowed to drain on absorbent material for 15-30 seconds and gently blotted to remove any droplets adhering to the rim before returning them to the upright position.
10. All tubes were counted in a gamma counter for one minute.

**Calculation of results**

The mean of duplicate determinations were calculated for all calibrators, samples and controls, and then the bound radioactivity (%B/B₀) was calculated from the following formula:

\[
%B/B₀ = \frac{(\text{calibrator or sample count}) \times 100}{\text{0 calibrator count}}
\]
A calibration curve was constructed and the oestradiol concentrations of the unknowns were determined from the standard curve (Figure 3.1) and expressed as pmol/L and then converted in pg/mL plasma.

![Oestradiol calibration curve](image)

**Figure 3.1** Oestradiol calibration curve

### 3.3.2 Measurement of metabolic parameters

In order to fully assess the condition of a liver, one must consider four groups of plasma metabolites: lipids, carbohydrates, proteins, and enzymes. Hence, plasma metabolites, such as cholesterol (CHOL), triglycerides (TG), total protein (TP), glucose (GLU), gamma glutamyl transferase (GGT), aspartate aminotransferase (AST) were determined. Not all metabolites were measured in all experiments; for each experiment important metabolites were targeted and measured in samples. To measure these metabolites in plasma, commercial kits and a chemistry system (VetTest chemistry analyser, IDEXX Laboratories, Inc. USA) were used. The instrument is based on dry chemical technology and colorimetric reaction. Dry-slide technology ensures that even compromised samples produce accurate results. Unique layers provide accurate results even on samples compromised by haemolysis or lipaemia (as it is the case of high levels of TG in avian female samples).

Plasma fibrinogen content was determined by heat precipitation method (Schalm, 1980) using an automatic temperature compensated refractometer with protein scale. This test was performed only on EDTA samples. The fibrinogen concentration was calculated by the difference between two plasma protein measurements (total plasma protein and plasma protein concentration after
heating/incubating at 50°C which precipitates the fibrinogen). The difference is equivalent to the fibrinogen that was removed from the plasma in the second tube by heating and centrifuging. Data collected from the refractometer were determined in g/dl then results were multiplied by 1000 to obtain the fibrinogen measurement in mg/dl.

### 3.3.3 Liver macroscopic evaluation

The liver was removed and individually examined for the presence of haemorrhagic lesions. Haemorrhages were counted on both the dorsal and ventral surfaces of the liver. Liver haemorrhages were graded on a scale from 0 to 5, with 0 indicating no haemorrhages; 1, up to 10 subcapsular petechial or ecchymotic haemorrhages; 2, more than 10 subcapsular petechial or ecchymotic haemorrhages; and 5, massive liver haemorrhage accompanied by rupture of the Glisson's capsule (Diaz et al., 1994). A haemorrhagic score of three to five was considered highly characteristic of FLHS (for details see Chapter 5.3.3). Two sections from the liver of each bird were dissected and used for histological tests. The remaining liver tissue was kept frozen (−20°C) for further lipid analysis.

### 3.3.4 Liver histology

Two slices of liver about 1 × 1 × 0.3 cm thick were taken from the right lobe of each hen, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H&E) stain prior to microscopic examination. For each section of liver, randomly located areas were assessed using a light microscope (40x and 100x). A digital camera connected to the microscope was used to take pictures. Fat content was assessed by evaluating the incidence of fat vacuoles inside and between hepatocytes. A fat vacuole was considered to be any non-staining area of cytoplasm with a sharply defined border. Inflammation was determined as the occurrence of focal infiltration with leukocytes and haemorrhage was determined according to the dilatation of arteries and veins, and focal infiltration of liver tissue with RBC.

### 3.3.5 Liver lipid content

The lipid content of liver was determined by the method of Folch et al. (1957). One gram of sample was weighed into a screw-capped test tube with 20 mL of chloroform/methanol (2:1, vol/vol), and homogenized with a polytron (Type PT 10/35) for 5 to 10 s at high speed. After an overnight incubation at 4°C, the homogenate was filtered through Whatman #1 filter paper into a 100-mL
graduated cylinder, and 5 mL of 0.88% sodium chloride solution was added and mixed. After phase separation, the volume of lipid layer was recorded, and the top layer was completely drained off. Total lipids were determined gravimetrically after evaporating the solvent. The sample was then dried and weighed, and the total lipid weight was calculated, and expressed as the percentage of liver fat against the total liver weight.

3.3.6 Quantitative analysis of cytokine gene expression in the liver

The levels of cytokine gene expression in the liver were analysed, focusing on mRNA expression levels of Interleukin (IL)-1β, IL-6, and IL-18. The mRNA determination was carried out using real-time qRT-PCR assays. Total RNA was extracted from samples using an RNeasy plus mini kit (Qiagen, Doncaster, VIC Australia), following the manufacturer’s directions. Liver samples were stored at –80°C prior to mRNA measurements. For details on the protocol and mRNA fold-change calculation see Chapter 7.2.3.

3.3.7 Haematological tests

Whole blood was used to measure haematological parameters in an automated analyser (CELL-DYN® System 3700CS, Abbott Park, IL 60064). Results obtained from the haematology analyser were used for the total number of red blood cells (RBC), white blood cells (WBC), packed cell volume or haematocrit (HCT) and haemoglobin (HGB) concentration. It was thought that these parameters would help to identify the presence of haemorrhage/haematoma in the liver or abdominal cavity. The RBC, HCT and HBG are decreased in haemorrhages and haematomas. The HCT is one of the most precise methods of determining the degree of dehydration and acute bleeding anaemia. The HGB should be evaluated with HCT and RBC to determine the type of anaemia.

3.3.8 Examination of the ovary and oviduct

At necropsy birds were examined for internal ovulations, internal oviposition, ovarian enlargement or regression and follicular atresia.
3.4 Data processing and statistical analysis

In this section, only a brief overview of the used statistical methods is described. A more specific description of the statistical analysis can be found in each chapter. Statistical analyses were processed using SAS/STAT software, Version 8 of the SAS System for Windows (SAS Institute Inc, 2001). For experiment 4 statistical analyses on cytokine gene expression measurement were performed using the two tailed Student’s t-test at the 99% confidence level (Microsoft Office Excel 2010 v14.0). A degree of significance of 5% was used in all experiments and data are shown as mean ± SEM, or SD as specified in the tables and figures. Significant differences among treatments/groups are mainly determined using Duncan’s multiple comparison tests, and correlations between different significant measures are determined using Pearson’s correlation coefficient. For parameters measured repeatedly in time from the same chickens, a statistical model for repeated measurements in time was used. Other specific statistical tests are described in the respective chapter.
CHAPTER 4

OCCURRENCE OF FLHS IN LAYING HENS KEPT IN DIFFERENT HOUSING SYSTEMS

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4.1 Introduction

Worldwide, around 85% of the laying hens are housed in the conventional cage system (Windhorst, 2011). In Australia, approximately 55% of eggs are produced in cage layer farms, with the balance coming from barn (9%) and free-range (34%) farms (AECL, 2011). It has been recognised that, typically, metabolic disorders such as FLHS cause loss to the industry through reduced productivity and increased mortality (Julian, 2005; Leeson, 2007; Scheele, 1997). Taking in consideration that more than 50% of Australia’s layer flocks are caged, the investigation of the effect of housing system on causes of mortality in laying hens is of economic and ethical importance for Australian poultry industry. In particular, reviewing an “old problem” for Queensland flocks such as FLHS was seen as a necessity.

It should be mentioned that nutritional factors (i.e., total M.E intake per day) and non-nutritional factors such as genetics (with some strains being more susceptible) have been seen as primary factors implicated in the aetiology of FLHS (Squires and Leeson, 1988). Nevertheless, in terms of space and microclimate conditions, housing system could be considered a very important factor,
because it may predispose hens to this metabolic disorder. It should be noted that the egg industry has progressively evolved from “as a side-line or by-product in small family farms” (Poultry Tribune, 1900) to modern egg farming when hens are kept in battery cage systems. Before 1940, small backyard flocks of chickens made up the majority of the egg producing industry worldwide, and the cage system began to spread in the mid-to-late 1940s (United Egg Producers, 2014).

As the poultry industry expanded in the 1950s, conventional cage egg production systems appeared to reduce disease and provided cleaner eggs. They proved to be more economically efficient as previous systems; the new systems were automated and more laying hens could be managed in less space (Mench et al., 2011). Over time, conventional cage systems have been heavily criticized for providing poor welfare for laying hens (Lay et al., 2011). Later on, many investigators started to demonstrate effects of cage housing of laying hens on bird health, and cage system housing was criticized as being a significant contributor to major metabolic diseases (Baxter, 1994; Craig and Swanson, 1994; Julian, 2005; Leeson, 2007; Scheele, 1997; Webster, 2004).

The first classical description of a metabolic disorder was that of cage layer fatigue, recognized in 1955 (Leeson, 2007; Webster, 2004). Over the past three decades, alternative housing, also known as the “free range, barn raised, or perchery” has been introduced to the egg industry and a progressive shift, from conventional to alternative housing, has been seen in the last 20 years (Appleby et al., 2002; Elson, 2010; Hester, 2005; Lay et al., 2011; Tauson, 1998). However, to a certain degree it appears that no single housing system is ideal from a hen welfare perspective (Lay et al., 2011). Currently modern poultry industry is represented by both conventional and alternative intensive housing, which can be responsible for different health problems that are characteristic for hens kept in cages or floor, respectively (Rodenburg et al., 2005; Shimmura et al., 2010; Shini et al., 2006; Weitzenburger et al., 2005).

The aim of this study was to collect data on mortality and mortality causes of hens kept in cages and alternative housing systems, and evaluate the impact of different layer housing conditions on the occurrence of FLHS, and general health and welfare of hens.
4.2 Materials and methods

4.2.1 Birds and bird husbandry

The investigation was carried out at the Gatton layer facility, University of Queensland. This facility provides a unique resource for investigations in terms of management practices used in different housing systems (conventional cages, barn, and free-range) on laying hen production, health, welfare and profitability.

Hy-Line Brown layers of the same hatch were housed under three different housing systems. Birds were reared on the same farm and under the same conditions as they were placed during the laying cycle (i.e. free-range birds were reared on range, barn birds reared in barns etc.), and subjected to the same diet, light and vaccination programs. Pullets were placed in the trial facilities at 17 weeks of age and fed a diet with the following specifications: CP - 17.5%; ME - 11.5 MJ/kg; Ca - 4.1%; Avail. P - 0.40%; Na - 0.18%; Lysine - 0.85%, Meth. + Cys.-0.77%. For details on diet see Appendix 2. Water and feed were provided ad libidum. Beak trimming was done at day old for all the caged layer hens and barn hens. In accordance with RSPCA recommendations, no beak trimming was done on the free range. Pullets had a constant 16:8 hour lighting program.

4.2.2 Housing systems

The free range system comprised three separated units located adjacent to each other which allowed a density of 5 hens/m2 of floor space or 600 hens per shed, 5 hens per nest boxes, 120mm of perch per hen, nipple drinkers and automatic feed chain feeders; hens had access to a grassed outdoor free-range area suitable for 1500 hens/hectare. The barn consisted of 3 pens each housing 600 hens at approximately 7 hens/m2 with 1/3 litter and 2/3 slatted floor with perches, automated nest box system, nipple drinkers and automatic feed chain feeders. Cages (Euro-vent, Big Dutchman, 6 hen/cage) were located in an environmentally controlled shed with tunnel ventilation and a pad system for summer cooling and fan assisted heaters for winter. Cages were in three tiers (3 replicates), with 48 units per tier, and in two rows back to back, holding 580 hens per replicate, and providing 550cm²/hen, and 2 x nipple drinkers/cage.
4.2.3 Determination of the mortality rate and mortality causes

Data on cumulative mortality were collected from 19 to 70 wk of age. Mortality rate was calculated as a percentage of the initial number of hens in a flock using the formula:

\[
\text{Mortality rate} = \frac{\text{Number of dead birds in a group}}{\text{Initial number of birds in the group}} \times 100
\]

Mortality causes were determined from gross necropsy of birds that died between 30 to 62 weeks of age. Histological, bacteriological, parasitological and other laboratory examinations were not performed in this study. Only gross pathological findings were used to determine the cause of death. All dead birds were necropsied according to a routine protocol (see Appendix 3). Birds that died (or appeared sick and were euthanised) were collected daily, refrigerated and necropsied weekly. The results given here are only for necropsied bird (240); not all dead birds recorded during this period were necropsied (in total 263). Some dead birds were not found (missed) or their body was highly decomposed therefore the necropsy was not conducted. The findings at necropsy were recorded individually for every hen and assigned to the appropriate housing system. Other problems such as parasites and other pathologies that did not lead to death of hens (or did not present any reason to euthanise hens) are not included or discussed in this study.

4.2.4 Performance parameters

Data on egg production and body weights (BW) were collected from 19 to 70 wk of age. Body weight was measured monthly. Fifty birds from each pen in the free-range and barn systems, and all birds in each cage (6 hens) from 9 cages (54 birds per replicate) were weighed at each time point. The average for each replicate was calculated and presented as the mean ± SD.

Eggs were collected daily between 09:30 and 12:30. The number of eggs laid in a flock is given as a percentage of hen-day production (%HDP) parameter, where hen-day is the number of hens alive in a house at a specific day over the whole observation period. Data on feed consumption were recorded inconsistently for all systems, and therefore not used in this study.
4.3 Statistics

Data were subjected to one-way ANOVA for repeated measure tests (with housing system as the main factor), and SAS program (SAS Institute Inc, 2001) was used to test for null hypothesis that the housing system does not have any effect on hen performance and mortality. For further interpretation of data, General Linear Model (GLM) procedures were used. When significant differences were found, comparisons among systems were conducted by Duncan’s multiple comparison tests. Statements of significance are based on \( P < 0.05 \). Correlations between BW and mortality rate, and BW and %HDP for birds housed in cages were determined using Pearson correlation coefficients. The means (n=3) were calculated for each system and presented in Figures and Tables.

4.4 Results

4.4.1 Mortality rates and necropsy findings

The average of mortality rate from 19 to 70 wk of age for hens housed in three different systems is presented in Table 4.1. The cumulative mortality at 70 wk of age and data on necropsy findings (i.e. causes of mortality) in conventional and alternative housing systems are presented in Table 4.2. At 70 wk of age no significant differences (\( P = 0.70 \)) were found in mortality rates between different housing systems. The mean of cumulative mortality rate from the start of lay until 70 wk of age was 6.1 %, 6.4 %, 5.8 %, respectively, for cages, barn and free range hens.

However, the gross pathology findings showed that causes of mortality for birds in different systems were different. More interestingly, the necropsy results indicated that, from birds that died and were necropsied in the cage system, 74% died due to FLHS. The condition was recognised from internal haemorrhage or haematoma due to liver capsule rupture. An excessive amount of fat was found in the abdominal cavity and surrounding the liver of these birds. There were several birds from the cage system (7% of all dead necropsied birds) that showed prolapse (but not signs of cannibalism), while 5% of examined birds were pecked and died due to cannibalism. One bird, which appeared sick was euthanised and had blocked eggs in the abdomen (egg binding), while in 6 cadavers the cause of death was unclear.
Table 4.1  Mortality rate and causes of death from necropsy findings in hens in different housing systems.

<table>
<thead>
<tr>
<th>Most common causes of death</th>
<th>Conventional</th>
<th>Barn</th>
<th>Free range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality rate (%)</td>
<td>6.1</td>
<td>6.4</td>
<td>5.8</td>
</tr>
<tr>
<td>N of birds examined</td>
<td>60</td>
<td>101</td>
<td>79</td>
</tr>
<tr>
<td>1. Cannibalism in total (%)</td>
<td>5</td>
<td>59</td>
<td>77</td>
</tr>
<tr>
<td>- Vent</td>
<td>75</td>
<td>23</td>
<td>27</td>
</tr>
<tr>
<td>- Body</td>
<td>25</td>
<td>66</td>
<td>70</td>
</tr>
<tr>
<td>- Head</td>
<td>0</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>2. Inflammation (%)</td>
<td>7</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>- Salpingitis</td>
<td>7</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>- Enteritis</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3. Rupture of liver and fatty liver (%)</td>
<td>74</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>4. Cachexia (%)</td>
<td>0</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>- Cachexia associated with ovarian inactivity</td>
<td>0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>5. Unclear cases (%)</td>
<td>11</td>
<td>17</td>
<td>9</td>
</tr>
</tbody>
</table>

1Cumulative mortality at 70 wk of age
Table 4.2  Egg production (%HDP) and mortality (%) in laying hens from 19 to 70 wk of age.

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>%HDP(^1)</th>
<th>Mortality (%)(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CE</td>
<td>FR</td>
</tr>
<tr>
<td>19</td>
<td>16.5</td>
<td>3.6</td>
</tr>
<tr>
<td>24</td>
<td>94.6</td>
<td>90.3</td>
</tr>
<tr>
<td>28</td>
<td>92.9</td>
<td>88.6</td>
</tr>
<tr>
<td>33</td>
<td>93.2</td>
<td>90.6</td>
</tr>
<tr>
<td>37</td>
<td>91.7</td>
<td>87.0</td>
</tr>
<tr>
<td>42</td>
<td>90.2</td>
<td>88.4</td>
</tr>
<tr>
<td>46</td>
<td>87.3</td>
<td>86.3</td>
</tr>
<tr>
<td>51</td>
<td>80.2</td>
<td>80.0</td>
</tr>
<tr>
<td>55</td>
<td>80.7</td>
<td>75.0</td>
</tr>
<tr>
<td>60</td>
<td>78.4</td>
<td>73.6</td>
</tr>
<tr>
<td>65</td>
<td>75.5</td>
<td>65.6</td>
</tr>
<tr>
<td>70</td>
<td>76.4</td>
<td>65.0</td>
</tr>
</tbody>
</table>

\(^1\)Each value represents the average of data recorded from each unit (replicates, n=3) calculated on the basis of number of hens at each unit at each time point.

In the barn system, the examination of dead hens showed that the cause of death in 59% of all cases was due to cannibalism. There were 16 birds (or 17% of birds examined) in which the cause of death was unclear. Abdominal haemorrhage was present in 5% of examined birds. Ten examined birds showed inflammation of the ovary, oviduct and cloaca (salpingitis), and one bird showed necrotic alterations of the intestine. In the free range, 77% of birds examined showed fresh lesions/wounds, and in some cases eaten parts of the muscles or cloaca and intestine, providing evidence of cannibalism. There were 11 birds (or 14% of examined birds) that died due to cachexia (no signs of cannibalism were found). Examination of the reproductive tract of cachectic birds showed the presence of an undeveloped ovary and lack of large yellow follicles. The cause of death was unclear in 7 cadavers from free range housing.

4.4.2  Performance parameters

Data on egg production (%HDP) are presented in Table 4.2, and data on BW for all systems are presented in Figure 4.1. Data are compared at 70 wk of age.
Egg production

As shown in Table 4.2, hens in cage system started laying eggs earlier than hens in alternative system, although their egg production peaked at a similar age. Most of hens achieved their peak of production at 33 wk of age (with %HDP over 90%), thereafter the egg production decreased. At the end of the laying period there were no significant differences in %HDP (P>0.05) among systems. Hens in all systems had a comparable %HDP ranging from 65% to 75% at 70 wk of age.

Body weights

Data on BW are presented in Figure 4.1. As shown from the line chart, all birds started the laying cycle with a similar BW (>1700g per bird). From the start to the peak of the laying period, BW was increased significantly (P<0.01) in all systems. All birds were heavier around 53 weeks of age; however at the end of 69 wk birds in cages continued to have BW over 2100g, while birds in barns and free-range dropped their BW approx. 100 to 200g/bird, respectively. For birds kept in the cage system, the highest body weight was achieved at 49 wk ca. 2200g/bird. At the end of 69 wk of age, the BW for hens in cages was significantly higher (P<0.001) than hens in free range and barns.

Correlation analysis were conducted to see if there were any relationships between variables measured, explicitly between the BW (which was the variable that showed a significant difference), and mortality rate or %HDP, and to measure the strength of this relationship. There was a positive and strong significant correlation between BW and mortality rate, and BW and %HDP in cage system (r=0.78, P=0.00014). There was a weak correlation between mortality rate and %HDP in this system (Table 4.3).
Figure 4.1  Monthly average of BW\(^1\)(g) in laying hens from 21 to 70 wk of age.

\(^1\)Each value represents the average (n=3) of each unit (replicate) calculated on the basis of number of hens that were weighed at each unit at each time point.

Table 4.3  Pearson’s Correlation of BW, mortality rate and %HDP for hens kept in cage system.

<table>
<thead>
<tr>
<th>Cage System</th>
<th>Mortality</th>
<th>BW</th>
<th>%HDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality rate</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW</td>
<td>0.78 (P&lt;0.01)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>%HDP</td>
<td>0.15</td>
<td>0.46 (P&lt;0.01)</td>
<td>1</td>
</tr>
</tbody>
</table>
4.5 Discussion

This study was undertaken to collect data on mortality and determine causes of death of hens kept in three different housing systems in a layer flock facility at Gatton Campus, University of Queensland. It was thought that recording data on mortality in three different housing systems along with data on egg production and body weights, it would help to not only assess the mortality in each system but also determine if the mortality rate is related to other performance parameters or any other problem associated with the housing system (i.e. husbandry practices and or environmental conditions associated with each system). Causes of mortality were determined through the necropsy of dead birds.

The results of this study showed that at the end of the lay period, there were no significant differences ($P>0.05$) in mortality rate among housing systems, and the mortality rate was comparable between all housing systems. In this case, the null-hypothesis that housing system does not affect mortality rate was confirmed. However data on causes of mortality showed that housing system had a major influence on the causes of hen death. The most common cause of death in hens kept in cages was FLHS with 74% of hens dying from this condition. The majority of hens kept in free range died from cannibalism (77%) and cachexia (14%), while hens in barns had problems such as cannibalism (59%), reproductive tract infections (10%) and cachexia from stress and malnutrition.

At 70 wk of age, %HDP was also comparable among the three systems, with hens in cages achieving the highest level of egg production at the peak (ca. 33 wk) and at the end of egg production (at 70 wk). However these differences were not statistically significant, confirming again the null-hypothesis, that there is no effect of the type of housing system on hen egg production.

Data on BW showed that, as hens got older, the average BW increased in all systems. Close to the end of egg production (at 65 and 69 wk of age) BW of hens in free range and barn system started to decrease; this wasn’t true for hen in cages, whose BW did not change or was slightly increased. It was concluded that housing system did affect the BW therefore the null-hypothesis was rejected.

Data on necropsy showed that the housing system influenced the causes of mortality, including the prevalence of FLHS. These data demonstrated that the highest incidence of FLHS happened in hens kept in cages. Previous investigators have suggested that FLHS is a metabolic disease of hens housed in conventional cages (Butler, 1976; Couch, 1956; Hansen and Walzem, 1993; Julian, 2005;
Leeson, 2007; Neill et al., 1975; Peckham, 1984; Ringer and Sheppard, 1963; Simonsen, 1978; Weitzenburger et al., 2005), and this statement was confirmed in this study. It was previously suggested that increased BW has a great impact on hen mortality and in most of cases is associated with fatty livers and FLHS (Harms et al., 1972; Pearson and Butler, 1978c; Schumann et al., 2003; Walzem et al., 1993). Moreover, one of the most obvious clinical signs of FLHS is overweight of hens (typically 25%) and, if associated with sudden death of hens, sudden drop in egg production and pale comb and wattles (Couch, 1956; Crespo and Shivaprasad, 2003; Ivy and Nesheim, 1973; Julian, 2005; Lumeij, 1994; Thomson et al., 2003), could be warning signs of the presence of FLHS in a flock.

As mentioned above, in this study, a high occurrence of FLHS in the cage system was related to an increased BW of hens. There was a high correlation between BW and mortality prevalence. It is possible that birds that died due to FLHS were heavier than other birds in the same cage/system, although data were not collected/not shown for BW of dead birds in this study. However, higher BW was associated with increased mortality in this system. As regards HDP, correlation data showed that this variable was also related to BW. Previous studies have demonstrated an increased incidence of FLHS in high producing flocks (Couch, 1956; Harms et al., 1972; Julian, 2005; Lee et al., 1975; Leeson, 2007; Squires and Leeson, 1988).

As shown by correlation analysis, there was a positive and highly significant correlation between BW and mortality rate, and BW and %HDP in the cage system. In contrast, there was a weak correlation between mortality rate and egg production indicating that the level of production was a lower contributor to the mortality rate, and subsequently the occurrence of FLHS in the cage system. At peak production (ca. 33 wk of age), the mortality rate was under 2%. Apparently due to an excess in energy balance, birds in the cage system accumulated more fat than birds in other systems, resulting in an increased BW in these birds. An increased BW is also related to the lack of physical activity of birds, which were unable to burn off the extra dietary energy. The haemorrhage presumably occurred due to fat accumulation in the liver and liver capsule rupture when hens straining to lay the egg. As a result, death occurred unexpectedly. The reasons and detailed events of liver rupture have been so far unknown, and will be investigated and discussed in this thesis.

From this preliminary investigation, it was suggested that there is a high incidence of FLHS in the cage system and BW of hens is an important factor that contributes to this incidence. Monitoring BW throughout the laying cycle appears to be a helpful diagnostic tool to predict FLHS occurrence in a laying flock. Meanwhile, it is suggested that, in the case of increased BW in a flock, attempts should be made to control and regulate the total energy intake of hens.
CHAPTER 5

FLHS OCCURRENCE IN QUEENSLAND COMMERCIAL LAYING FLOCKS

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5.1 Introduction

Fatty liver haemorrhagic syndrome is a production disorder and an important cause of mortality in caged commercial layer flocks, with obvious welfare and economic implications (Craig and Swanson, 1994; Diaz et al., 1999; Leeson, 2007). Since the 1950s, a number of investigations have been conducted into the aetiology and prevention of this disease, but the aetiopathogenesis is still not completely understood. Currently it is seen as an unresolved problem in laying hens, and anecdotally is called the “silent killer.” There are no recent data available on the incidence of FLHS in Australia. Neill et al. (1975) were the first to report outbreaks of FLHS in laying hen flocks in South East Queensland, Australia. Thirty years later, Shini et al. (2006) examined the causes of mortality of laying hens in a flock from the same region, and showed that hens kept in battery cages had a higher occurrence of FLHS. More specifically in the case of a mortality rate of 5%, more than 70% of deaths were due to FLHS.
As FLHS is often asymptomatic, most producers are unaware of its occurrence in their flocks. The first sign of the syndrome is a sudden drop in egg production and an increase in mortality, with birds in full production being found dead. In many cases, affected hens are overweight with pale combs and wattles; however diagnosis of FLHS can only be confirmed at necropsy (Grimes et al., 1991; Squires and Leeson, 1988). Haemorrhage occurs when the hen strains to lay an egg or from trauma resulting in the liver blood vessels rupturing (Crespo and Shivaprasad, 2003). The liver at necropsy appears enlarged, yellowish, and friable. In some cases subscapular haemorrhage and several hematomas are found inside or on the surface of the liver with or without internal blood clotting (Crespo and Shivaprasad, 2003). Large blood clots and excessive fat deposits are found in the abdominal cavity.

Outbreaks of FLHS occur sporadically in commercial flocks (Hoerr, 1996; Squires and Leeson, 1988), with a mortality rate of 3-5% (Dimitrov et al., 1980; Hansen and Walzem, 1993; Pearson and Butler, 1978c; Valkonen et al., 2008) although higher mortality rates have been reported (Shini et al., 2006; Ugochukwu, 1983; Weitzenburger et al., 2005). Industry estimates suggest that between one third to two thirds of caged layer mortality can be attributed to FLHS. However, to date there is no husbandry strategy applied in laying flocks that can prevent FLHS, most probably due to the fact that the aetiology of this syndrome is poorly understood and the occurrence probably underappreciated. It should be stressed that both decreases in egg production and increases in mortality associated with FLHS have implications for hen welfare and cause considerable economic loss to the egg industry in Australia. Since about 55% of Australia’s commercial layer flocks are housed in cages, FLHS is a disorder of major importance.

From the results of the first study in this thesis, it was suggested that there is a need to survey more flocks in Queensland and re-evaluate the situation after 30 years; this is required due to changes in many husbandry procedures and especially developments in terms of laying hen breed, nutrition and housing.

5.2 Objectives

The objectives of this study were to:

(i) Determine the occurrence of FLHS in commercial caged layer flocks located in the South East Queensland of Australia, and

(ii) Ascertain the importance of some husbandry factors in predisposing hens to this condition.
5.3 Survey design

This region of Queensland had approximately 2.933 million laying hens (AECL, 2011). The epidemiological study involved both a questionnaire sent to 20 registered cage layer operations in Queensland, and a four-month survey to monitor health and production of selected farms or flocks from these operations. Of the farms that responded to the questionnaire, three farms (designated farms 1, 2 and 3) were selected for an on-farm flock epidemiological survey (Table 5.1). All of the procedures undertaken in this study were approved by the University of Queensland Animal Ethics Committee (SAS/871/07).

5.3.1 Questionnaire

The aim of the questionnaire was to collect data on layer bird management, health and productivity. The questionnaire contained questions on breed and flock age, feed source, lighting program, egg production, body weight (BW) monitoring, health (including vaccination), mortality, and the use of laboratory tests and necropsy for determining causes of mortality. Producers were also asked if they were able to recognize deaths FLHS, and if they could estimate its occurrence in their flocks (for more details see Appendix 4).

5.3.2 Epidemiological studies on selected flocks

Farms 1, 2 and 3 were chosen for the epidemiological study because they were representative of the egg industry in Queensland, with respect to bird strain in use, size and age of flocks (start, middle, or end of laying cycle), and feed management (commercial vs. farm-mixed feed). For more details on feed and feed ingredients used in each Farm see Appendix 1 (Tables 1, 2 and 3). Data comparing laying hen nutrient levels as recommended by breeders until 44 wk of age (Australian ingredients) and provided in the diets used in farms surveyed are presented in Appendix 1 Table 4. Selection of the farms was stratified across geographic location (East and West from University of Queensland, Gatton Campus). A description of the farms is given in Table 5.1. It should be noted that only 7 flocks from these farms were surveyed in all; 3 flocks from each in Farms 1 and 3, and 1 flock in Farm 2. These flocks had a combined total of 21,903 laying hens. The farms were visited on three occasions over a 4-month period. Necropsies were conducted on farm and blood samples were collected for haematology and biochemistry. Data on mortality and mortality causes, egg production and BW were recorded over the monitoring period (4 months). Cases of FLHS were confirmed by lesions found at necropsy.
Table 5.1 Description of farms that participated in the epidemiological survey.

<table>
<thead>
<tr>
<th>Farm ID</th>
<th>Location from Gatton</th>
<th>Breed and strain</th>
<th>Age(^1) (wk)</th>
<th>Type of feed in use</th>
<th>Lighting (h)</th>
<th>BW monitoring</th>
<th>System, number of birds/cage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm 1</td>
<td>South-west</td>
<td>Hy-Line Brown</td>
<td>17</td>
<td>Farm-mixed</td>
<td>16</td>
<td>Yes, monthly</td>
<td>Environmentally controlled shed; multi-tier cages; 6 birds/cage at 550 cm(^2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>61</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm 2</td>
<td>West</td>
<td>Hy-Line Brown</td>
<td>60</td>
<td>Farm-mixed</td>
<td>16</td>
<td>Yes, monthly</td>
<td>Naturally controlled shed; single-tier cages; 3 birds/cage at 650 cm(^2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm 3</td>
<td>East</td>
<td>ISA Brown</td>
<td>19</td>
<td>Commercial</td>
<td>17</td>
<td>No</td>
<td>Naturally controlled shed; single-tier cages; 3 birds/cage at 550 cm(^2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Age at the start of monitoring
5.3.3 Sampling

**Performance parameters**

Production and mortality data were provided by farmers and used to calculate percentage hen-day egg production (%HDP), and percentage mortality rates expressed as cumulative mortality from the start of lay.

**Body Weights**

Each farm was visited once a month for 2-3 days to conduct necropsies, monitor BWs and collect blood samples. The same birds, taken at 3 different spots in the shed, were weighed on 3 separate occasions. For an accurate BW monitoring in layers, most breeder companies recommend individually weighing 3 to 5% of the flock at each weighing point. Others suggest that 2% of the flock is sufficient, provided that a minimum of 50 birds are weighed irrespective of flock size (Leeson and Summers, 2000). For Farm 1, with ca. 5,000 birds per flock, individual birds in 40 cage units (240 birds or 4.8% of birds per flock) were weighed; for Farm 2, with ca. 3240 birds per flock, birds in 48 cage units (144 birds or 4.4% of birds) were weighed; for Farm 3, with ca. 1500 birds per flock, birds in 24 cage units (72 birds or 4.8% of birds per flock) were weighed. Individual birds were weighed and the results are expressed as an average BW (g) per bird.

**Post-mortem examinations**

All dead birds from farms that participated in the survey were collected and mortality recorded. Due to insufficient freezer storing space, post-mortem was only carried out on birds that died during the first 10 days of each month for 4 months (from January to May). Dead birds were weighed and a thorough necropsy was carried out on each bird (for details on necropsy examination see Appendix 3). Particular attention was paid to the presence of excess amount of fat and blood clots or coagulations in the coelomic cavity. The liver was carefully removed, examined for haemorrhages and haematomas, and weighed. Haemorrhage was assessed on both the dorsal and ventral surfaces of the liver and was graded on a scale from 0 to 5, with score 0 indicating no haemorrhages; score 1, up to 10 subcapsular petechial or ecchymotic haemorrhages; score 2, more than 10 subcapsular petechial or ecchymotic haemorrhages; and scores 3-5, large haematomas and massive liver haemorrhage accompanied by rupture of liver capsule. A haemorrhagic score of 3-5 was considered highly characteristic of FLHS and was diagnosed as the cause of death (Figure 5. 1a, b, c, d, and e).
Figure 5.1 Pictures from post-mortem examinations of hens showing various stages of haematomas and haemorrhages scored 1 to 5 (a, b, c, d and e).
**Blood parameters**

The number of birds used for blood tests was calculated according to epidemiological formulas and recommendations by Australian Animal Health Laboratory (AAHL, CSIRO).

A minimum of 10 birds and a maximum of 50 birds per flock in a flock with 1,000 to 5,000 birds (or 1% of the flocks) were sampled, respectively. Random samples of birds were selected, and the results from the sample then provided an estimate of the status of the whole flock. The same bird was sampled for repeated measurements.

Blood samples for haematology and biochemistry were collected from the brachial vein on all occasions (see Chapter 3.3.1 and 3.3.6 for more details). Haematological parameters, such as total red blood cell (RBC) numbers, haematocrit (HCT) and haemoglobin (Hb) concentration were measured using an automated analyser (CELL-DYN® System 3700CS, Abbott Park, IL 60064). Blood for biochemistry was centrifuged (1500 rpm for 10 min), the plasma stored at -20ºC and subsequently analysed for CHOL, TG, TP, GLU, and GGT using commercial kits and a chemistry system (Vet Test chemistry analyser, IDEXX Laboratories, Inc. USA).

### 5.4 Statistical analysis

Valid responses from the questionnaires were used to record data on bird and farm management and evaluate the frequencies and percentages of all observations. They provided an estimate, but they were not suited to statistical analysis. For the flock epidemiological survey, it was acknowledged that a direct comparison of the data of 7 flocks from different farms would not be valid due to specific conditions and variables associated with one management system compared to another. Results from each farm were summarized into three periods (for each point of sampling) and used to evaluate trends in mortality and egg production, and compare BW, and blood health profiles within the flock for the period of study. Farms or flocks were not statistically compared with each other in any of the analyses. Recorded data were subjected to one-way ANOVA. All analyses were performed using the GLM procedure of the SAS version 8.0 software (SAS Institute Inc, 2001). Significant differences among sampling points were determined using the Tukey’s HSD (Honestly Significant Difference) test. Statements of significance were based on $P<0.05$. For data on BW and blood profile, the mean, standard error, and $P$ values are presented.
5.5 Results

5.5.1 Questionnaire

Eleven of the cage egg producers in Queensland (or 55%) replied to the survey, and their total number of flocks and hens was 40 and 1,384,500 respectively. They represented different geographic locations and had been operational for more than 20 years, with four farms for more than 50 years. Tables 5.2 and 5.3 present data on-farm and bird management recorded from the questionnaires. The average number of birds per farm per year ranged from less than 10,000 (2 producers), 20,000 to 190,000 (8 producers), and 900,000 birds (1 producer), with the number of sheds ranging from 1 to 16 per farm. Only two producers housed birds in environmentally controlled sheds, while the remainder of birds were maintained in naturally ventilated sheds. Seven of the producers (or 64%) used a cage system housing 5 or 6 birds/cage, and 4 producers used cages holding 3 birds/cage. All cages from surveyed farms complied with the Australian Model Codes of Practice for the Welfare of Animals (ARMCANZ and PISC, 2002).

Five producers (or 45.5%) had Hy-Line Brown egg hens, two (18.2%) had Isa Brown hens, three (27.3%) had Hisex brown hens, and one (9%) had both Hy-Line and Isa Brown strain hens. Seven of the producers mixed feed on farm, and only four purchased commercial feed. The mortality rate of flocks ranged from 2% to 11% and the average rate of production for laying cycle ranged from 70 to 89% (Table 5.3). Most of the producers did not know the causes of mortalities in their flocks, and only three of them used veterinary laboratories to determine the cause of mortality. Six (54.5%) producers monitored BW of their flocks, while all used lighting programs for flocks. Only one producer was aware of FLHS being present in their flock from necropsies conducted sporadically by a health adviser.
Table 5.2  Questionnaire data summary (farm data).

<table>
<thead>
<tr>
<th>Farm</th>
<th>N of sheds/birds(^1)</th>
<th>System, cage type &amp; number of hens/cage</th>
<th>Shed temperature (^2) (ºC)</th>
<th>Type of feed in use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/120K</td>
<td>Controlled environment/Valli/6</td>
<td>23-25</td>
<td>Farm-mixed</td>
</tr>
<tr>
<td>2</td>
<td>3/10K</td>
<td>Natural/Chainex/3</td>
<td>25-33</td>
<td>Farm-mixed</td>
</tr>
<tr>
<td>3</td>
<td>1/9.5K</td>
<td>Natural/Single tier/3</td>
<td>NA</td>
<td>Commercial/Darwalla</td>
</tr>
<tr>
<td>4</td>
<td>7/190K</td>
<td>Natural/Multi-tier/4&amp;6</td>
<td>25</td>
<td>Farm-mixed</td>
</tr>
<tr>
<td>5</td>
<td>1/10K</td>
<td>Natural/old cages/3</td>
<td>NA</td>
<td>Commercial</td>
</tr>
<tr>
<td>6</td>
<td>1/8K</td>
<td>Natural/Tier-Sylvan/5</td>
<td>15-34</td>
<td>Commercial</td>
</tr>
<tr>
<td>7</td>
<td>2/20K</td>
<td>Natural/multi-tier/6</td>
<td>25-30</td>
<td>Farm-mixed</td>
</tr>
<tr>
<td>8</td>
<td>2/18K</td>
<td>Natural/square old colonial wire/3</td>
<td>28-30</td>
<td>Commercial/Riverina</td>
</tr>
<tr>
<td>9</td>
<td>16/900K</td>
<td>Controlled-environment/Big Dutchman/6</td>
<td>21-28</td>
<td>Farm-mixed</td>
</tr>
<tr>
<td>10</td>
<td>2/74K</td>
<td>Natural/Techno &amp; Harrison/5</td>
<td>NA</td>
<td>Farm-mixed</td>
</tr>
<tr>
<td>11</td>
<td>1/25K</td>
<td>New cage system/6</td>
<td>22-23</td>
<td>Farm-mixed</td>
</tr>
</tbody>
</table>

\(^{1}\)Number of sheds and birds at the time of survey (1000=1K)  
\(^{2}\)During summer months  
NA = data not available
Table 5.3  Questionnaire data summary (bird data).

<table>
<thead>
<tr>
<th>Farm</th>
<th>Breed</th>
<th>Age of flocks(^1) (wk)</th>
<th>Average mortality (%)</th>
<th>Causes of mortality who determines</th>
<th>Average production (%)</th>
<th>Weighing program</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hy-Line Brown</td>
<td>17 to 61</td>
<td>4</td>
<td>unknown/ manager</td>
<td>85</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>Hy-Line Brown</td>
<td>60</td>
<td>2</td>
<td>unknown/ manager 90%</td>
<td>70</td>
<td>yes/ monthly</td>
</tr>
<tr>
<td>3</td>
<td>ISA Brown</td>
<td>19 to 52</td>
<td>7</td>
<td>unknown/ owner</td>
<td>70</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>Hy-Line Brown</td>
<td>47</td>
<td>2</td>
<td>unknown/ manager 95%</td>
<td>85</td>
<td>yes</td>
</tr>
<tr>
<td>5</td>
<td>ISA Brown</td>
<td>various</td>
<td>6</td>
<td>unknown/ owner</td>
<td>80</td>
<td>no</td>
</tr>
<tr>
<td>6</td>
<td>HI-SEX Brown</td>
<td>various</td>
<td>8</td>
<td>unknown/ owner</td>
<td>76</td>
<td>no</td>
</tr>
<tr>
<td>7</td>
<td>HI-SEX Brown</td>
<td>various</td>
<td>5</td>
<td>unknown/ manager</td>
<td>85</td>
<td>no</td>
</tr>
<tr>
<td>8</td>
<td>Hy-Line Brown</td>
<td>NA</td>
<td>11</td>
<td>unknown/ manager</td>
<td>80</td>
<td>no</td>
</tr>
<tr>
<td>9</td>
<td>Hy-Line Brown &amp; ISA Brown</td>
<td>various</td>
<td>2.1</td>
<td>unknown/ manager</td>
<td>80</td>
<td>yes/ monthly</td>
</tr>
<tr>
<td>10</td>
<td>Hy-Line Brown</td>
<td>various</td>
<td>10</td>
<td>unknown/ manager</td>
<td>82</td>
<td>no</td>
</tr>
<tr>
<td>11</td>
<td>HI-SEX Brown</td>
<td>various</td>
<td>2</td>
<td>unknown/ manager 50%</td>
<td>89</td>
<td>yes/ quarterly</td>
</tr>
</tbody>
</table>

\(^1\)Age of birds at the time of survey  
NA = data not available
5.5.2 Results from the epidemiological study

Data are presented from samples and records collected in 3 designated farms (Farm 1, 2, and 3).

Mortality and necropsy findings

Table 5.4 shows cumulative mortality (as monitored by producers) and the number of dead birds and necropsies conducted on all three farms during the study. The mortality rate of flocks ranged from 0.8 % (the youngest flock) to 11.6 % the oldest one. The mortality rates increased with age (P<0.001). The age trend in mortality rates for three flocks in Farm 1 is presented in Figure 5.2. Each part of the graph represents one flock, and the entire graph represents three flocks from the same farm jointly. The graph indicates that at 29, 54 and 73 wk of age the % cumulative mortality of flocks (1, 2 and 3) was 2, 4.8 and 11.6%, respectively. There was a higher mortality rate than expected for flock 1 at 73 wk of age, reflecting mortality at the beginning of laying period (according to the producer’s records and comments). The monthly mortality rate for these flocks during the study ranged from 1 to 1.2% per month. At 72 wk, for Farm 2 (flock 1) the mortality was calculated at 7.4% of the initial flock, and for Farm 3 at 31, 49 and 64 wk of age (flocks 1, 2, and 3) the mortality rate was 0.8, 2.5 and 4.8 %, respectively (Table 5.4).

As indicated in the methodology, only 30-50 % of dead birds were necropsied. At Farm 1, 42% of birds necropsied showed large subcapsular haemorrhage of liver and blood clotting in the abdominal cavity indicative of FLHS, while in Farm 2 and 3, 28 % and 34% of dead birds had signs of FLHS, respectively (Table 5.4). From birds that died in Farm 1, and were aged between 42 to 54 wk of age, about 63% demonstrated FLHS. For this study BW of all necropsied hens were recorded, hens with parts of the body missing were excluded from the calculations of BW average and statistical analyses. The BW average of dead birds in Farm 1 was 2008±107g. The average BW of birds that died in Farm 2 and 3 was 1821±78 and 1954±92g, respectively.
Table 5.4  Mortality (%), number of post-mortems conducted, and frequency of FLHS in three surveyed farms.

<table>
<thead>
<tr>
<th>Farm/ flocks surveyed</th>
<th>Flock size and age(^1) Number of birds (3 flocks)</th>
<th>Mortality (cumulative from 18 wk) Age (wk)</th>
<th>Number of dead birds during study</th>
<th>Number of post-mortems conducted during the study</th>
<th>Incidence of FLHS (number of birds and %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5022</td>
<td>29</td>
<td>2.0</td>
<td>619</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td>4776</td>
<td>54</td>
<td>4.8</td>
<td>579</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>4347</td>
<td>73</td>
<td>11.6</td>
<td>407</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>=14145</td>
<td>=1605</td>
<td></td>
<td>=482</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(30% of dead birds)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3240</td>
<td>72</td>
<td>7.4</td>
<td>121</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>(1 flock)</td>
<td></td>
<td></td>
<td>(ca. 50% of dead birds)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1635</td>
<td>31</td>
<td>0.8</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>1539</td>
<td>39</td>
<td>2.5</td>
<td>75</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>1344</td>
<td>64</td>
<td>4.8</td>
<td>118</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>=4518</td>
<td>=215</td>
<td></td>
<td>=105</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(50% of dead birds)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Age at the end of survey
Figure 5.2  Data on mortality for Farm 1: flock 1 (a), flock 2 (b) and flock 3 (c).
Mortality is presented only for the period of study as a percentage (cumulative) mortality of birds housed in the shed at start of lay.

Performance parameters

Table 5.5 shows data on hen performance (%HDP, BW and cumulative mortality) from three farms, and for comparison, data from respective layer breeding companies as recommended in their management guides (HY-Line, 2008; ISA, 2000). Body weight increased with age especially for birds in Farm 2 and 3 (Figure 5.3; data shown only for one flock per Farm at age ranging from 56 to 73 wk of age). However, this increase was not statistically significant ($P >0.05$) for the sampling period in three flocks. At 73 wk of age, there was a decrease in BW of birds from Farm 1, but again this difference was not statistically significant. At 29 and 69 wk of age, birds in Farm 1, and at 32 and 72 wk of age, birds in Farm 2, had a BW comparable or lower than that recommended by breeders (Table 5.5). There was a significant increase ($P < 0.05$) in BW recorded for hens in Farm 3 flock 3 from 56 to 64 wk of age. At 31 and 64 wk of age, birds in Farm 3 weighed more than breeder’s recommendations for this age (Table 5.5), data shown only for 64 wk of age.
Table 5.5  Comparative data on performance parameters as recommended by breeders in the management guides (HY-Line, 2008; ISA, 2000) and data monitored in three farms.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HY-Line Brown(^1)</th>
<th>Farm 1(^2)</th>
<th>Farm 2</th>
<th>ISA Brown(^1)</th>
<th>Farm 3(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32 wk</td>
<td>1980</td>
<td>1872</td>
<td>-</td>
<td>1885</td>
<td>1985</td>
</tr>
<tr>
<td>72 wk</td>
<td>2250</td>
<td>2128</td>
<td>2117</td>
<td>1985 (1975 at 64 wk)</td>
<td>2163</td>
</tr>
<tr>
<td>HDP (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32 wk</td>
<td>94</td>
<td>94.3</td>
<td>-</td>
<td>94.3</td>
<td>91</td>
</tr>
<tr>
<td>72 wk</td>
<td>72</td>
<td>77.4</td>
<td>74</td>
<td>75 (79.7 at 64 wk)</td>
<td>85</td>
</tr>
<tr>
<td>Mortality cumulative (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32 wk</td>
<td>0.8</td>
<td>2.0</td>
<td>-</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>72 wk</td>
<td>4.0</td>
<td>11.0</td>
<td>7.4</td>
<td>5.8 (4.9 at 64 wk)</td>
<td>4.8</td>
</tr>
</tbody>
</table>

\(^1\)At peaking (32 wk) and end of lay (72 wk);  
\(^2\)At 29 and 69 wk of age;  
\(^3\)At 31 and 64 wk of age

The age trend in %HDP for three flocks in Farm 1 is presented in Figure 5.4. From the start of lay until the peak the %HDP increased \((P < 0.001)\) with age. From 26 wk of age birds had a %HDP close to 90% and this continued at this level or above until 42 wk of age. At 64 wk of age, %HDP was between 82-85% in all farms. In general, the %HDP was comparable with the breeder’s recommendations for the respective age (Table 5.5), while at 64 wk of age birds in Farm 3 had a higher %HDP than that recommended by the breeder (85% vs. 79.7%) at this age.
Figure 5.3  Average body weight measurements for Farms 1, 2 and 3.
Data are shown here are only from 1 flock per farm and are presented as mean ±SD of hens weighed at each sampling point (every 4 weeks). Data were compared only within the same flock. Number of birds weighed at each sampling point was: Farm 1 (n=240), Farm 2 (n=144) and Farm 3 (n=72).

Figure 5.4  Data on egg production for Farm 1, flock 1 (a), flock 2 (b) and flock 3 (c).
Hen day production = HDP;
Data shown only for the period of study for each flock.
Blood parameters

Haematology and biochemistry parameters of hens from Farm 1 (flock 1 and 3), Farm 2 (flock 1) and Farm 3 (flock 1) are presented in Table 5.6, respectively. Data presented show measurements at 3 sampling points and are tabulated according to the age for visual comparisons. However, as mentioned in section 5.4 of this Chapter (statistical analysis) there were no statistical comparisons between the farms or flocks of different farms. Only data from repeated measurements of the same flock of each farm were used for statistical analysis.

From 21 to 29 wk of age, birds in Farm 1, flock 1, showed a significant increase in TG ($P<0.005$) and GLU ($P<0.001$) concentrations. Plasma concentration values peaked at 29 wk (17.9 mmol/L and 15.1 mmol/L for TG and GLU, respectively). These values were similar to plasma concentrations of birds in Farm 2, flock 1 at 31 wk of age (18.1 mmol/L and 15.1 mmol/L for TG and GLU, respectively). In older birds, Farm 1, flock 3 (at 73 wk of age), the TG levels were significantly higher (ranging 21 to 23 mmol/L) than in younger hens (Farm 1 flock 1 at 29 wk of age). Glucose levels were found similar in birds of different flocks; there was a slight significant change in GLU levels in birds of the same flock over time. There were no significant changes between the values of the CHOL and TP levels measured in birds of different flocks and ages.

The GGT and TG levels increased ($P<0.001$) in birds in Farm 3, flock 1 at 68 wk of age, and then both values significantly ($P<0.001$) decreased at 72 wk. This was associated with a decrease in egg production. In contrast, total plasma protein levels increased ($P<0.001$) in these hens. The TG levels in all young flocks on all Farms showed similar patterns. The concentration was increased over time and peaked at 35 weeks of age (18.1 mmol/L) and thereafter in older birds was found ranging 20 to 23 mmol/L. There were no significant changes in RBCs, HGB, and HCT in hens of different flocks or ages on Farms 1, 2 and 3 (Table 5.6) except for a slight increase ($P<0.05$) in HCT in birds in Farm 1 flock 3 at 73 wk of age.
### Table 5.6  Plasma metabolites and haematological profiles of birds (Farm 1, 2 and 3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Farm 1 Flock 1 (21 to 29 wk)</th>
<th>Farm 2 Flock 1 (23 to 31 wk)</th>
<th>P-value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21 wk 25 wk 29 wk</td>
<td>23 wk 27 wk 31 wk</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>2.84 ±0.10 3.30 ±0.04 2.77 ±0.03</td>
<td>2.70 ±0.10 2.96 ±0.21 2.48 ±0.03</td>
<td>0.255</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.115</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>15.3 ±0.59 16.7 ±0.21 17.9 ±0.20</td>
<td>15.2 ±0.54 14.1 ±0.32 18.1 ±0.22</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>12.2 ±0.43 14.9 ±0.18 15.1 ±0.18</td>
<td>12.5 ±0.18 14.7 ±0.05 15.1 ±0.19</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/L)</td>
<td>43.7 ±3.52 51.7 ±0.80 47.8 ±0.18</td>
<td>43.5 ±2.80 50.6 ±2.10 48.7 ±1.20</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.102</td>
</tr>
<tr>
<td>GGT (IU/L)</td>
<td>33.7 ±3.76 34.0 ±1.53 32.2 ±1.64</td>
<td>37.0 ±1.73 32.7 ±2.33 35.0 ±3.51</td>
<td>0.960</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC (x10^6/L)</td>
<td>2.38 ±0.09 2.47 ±0.05 2.59 ±0.147</td>
<td>2.41 ±0.25 2.49 ±0.07 2.51 ±0.11</td>
<td>0.073</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>131 ±3.17 135 ±3.29 135 ±2.88</td>
<td>133 ±4.91 134 ±3.08 133 ±4.56</td>
<td>0.965</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT (L/L)</td>
<td>0.31 ±0.01 0.30 ±0.01 0.32 ±0.01</td>
<td>0.29 ±0.01 0.30 ±0.01 0.30 ±0.01</td>
<td>0.740</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parameter</td>
<td>Farm 1 Flock 3 (65 to 73 wk)</td>
<td>Farm 3 Flock 1 (64 to 72 wk)</td>
<td>P-value²</td>
</tr>
<tr>
<td></td>
<td>65 wk 69 wk 73 wk</td>
<td>64 wk 68 wk 72 wk</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>2.65 ±0.01 2.58 ±0.01 3.09 ±0.01</td>
<td>2.34 ±0.05 2.20 ±0.02 2.44 ±0.03</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>21.1 ±0.23 21.0 ±0.33 23.2 ±0.94</td>
<td>23.5 ±0.57 24.5 ±0.29 20.1 ±0.29</td>
<td>0.073</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>12.9 ±0.34 14.5 ±0.28 15.7 ±0.07</td>
<td>13.4 ±0.07 14.1 ±0.11 13.0 ±0.10</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/L)</td>
<td>57.0 ±1.20 55.3 ±1.50 56.0 ±2.00</td>
<td>5.52 ±0.06 5.24 ±0.12 6.04 ±0.11</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGT (IU/L)</td>
<td>45.0 ±1.53 43.6 ±2.33 46.0 ±2.65</td>
<td>44.0 ±0.92 44.1 ±0.93 38.3 ±0.92</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC (x10^6/L)</td>
<td>2.62 ±0.08 2.52 ±0.05 2.69 ±0.03</td>
<td>2.28 ±0.03 2.35 ±0.02 2.39 ±0.05</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>139 ±3.77 139 ±3.50 148 ±0.80</td>
<td>121 ±1.59 126 ±1.95 127 ±1.83</td>
<td>0.093</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT (L/L)</td>
<td>0.33 ±0.01 0.32 ±0.01 0.34 ±0.01</td>
<td>27.4 ±0.39 28.4 ±0.44 28.7 ±0.54</td>
<td>0.048</td>
</tr>
</tbody>
</table>

¹Values in bold are significantly different; Each value is the average of n=50 (Farm 1), n=30 (Farm 2), and n=48 (Farm 3). Only selected flocks are presented.
5.6 Discussion

In this study, a questionnaire followed the monitoring of selected laying flocks, were used to study the epidemiology of FLHS in South East Queensland, Australia. The response rate to the questionnaire was 55%, and was considered satisfactory (Williams, 2003). The questionnaire provided important data on hen management practices, and also suggested that most egg producers are not aware of FLHS. However, due to the small number of participating farms and varying husbandry practices and procedures used on these farms, it was difficult to draw firm conclusions in relationship to management and the incidence of FLHS. The data from the intensive flock survey (on-farm systematic monitoring and sampling for 4 months) were used to evaluate and confirm the presence of FLHS in commercial layers flocks in Australia.

The presence of FLHS in this epidemiological study was confirmed only after the post-mortem monitoring in three farms. Necropsies conducted in 7 flocks of different ages indicated that of all birds examined, 39.3% (or 256 out of 651 necropsied birds) died due to FLHS, indicating that FLHS was a significant cause of death of laying hens on the surveyed farms. These results are in agreement with previous Australian and overseas findings which have shown that FLHS is one of the main causes of death in caged flocks irrespective of mortality rate (Grimes, 1975; Lee et al., 1975; Neill et al., 1975; Peckham, 1984; Rodenburg et al., 2005; Ugochukwu, 1983; Valkonen et al., 2008; Weitzenburger et al., 2005). It should be noted that not every dead bird was examined and in addition to this, only hens that were presented with large hematomas and massive liver haemorrhage were diagnosed as dying from FLHS. Therefore the actual prevalence of FLHS could be higher than reported in this study. It is well recognized that sample size required for such surveys depends on the prevalence of the syndrome studied. In the current study, to calculate the minimum number of birds necropsied from each flock at each sampling point, the occurrence rate was predicted to be as previously found by other in field investigations i.e., between 20% to 50% of dead birds (Butler, 1975; Couch, 1956; Dimitrov et al., 1980; Ugochukwu, 1983; Valkonen et al., 2008; Weitzenburger et al., 2005). Therefore, a minimum sample size of 14 necropsied birds was found to be sufficient to detect FLHS in a flock with a population of >1000 birds, for a prevalence estimate of disease 20% or more (with a confidence level 95%) (Cannon, 1982). In this study over 600 birds were necropsied and they represented over 40% of all birds that died in 3 farms during the epidemiological study (over 4 months), and from these birds ca. 40% revealed typical signs of FLHS. This level of occurrence is lower than the level found in the first study (when comparing housing systems), however is still very high in terms of the number of the birds examined (40% of dead birds) and the period of examination (3 times during a 4 month period), suggesting a much
higher prevalence during a full laying cycle. These data indicate that FLHS continues to be a disease with high prevalence in cages, most probably due to a restricted movement of hens in cages. Three experiments, (Greuel and Hartfiel, 1968; Hartfiel et al., 1972) investigated the effect of the housing system on the liver fat content, and concluded that the percentage of hens with a relatively high liver fat content decreased when hens were kept on the floor. Moreover, they indicated that a limited opportunity for hens to move in cages was one of the factors which increased liver fat content.

The results of this study indicated that laying hens in multi-tier cages in a controlled environment shed (as exposed in Farm 1), are at the same risk of developing FLHS as hens kept in naturally controlled sheds (Farms 2 and 3). This is the first demonstration of a similar effect of a thermo-neutral environmental temperature on the occurrence of FLHS in caged hens. Earlier studies that examined the effect of temperature on the occurrence of FLHS were conducted some 20 to 30 years ago, when controlled environment sheds were not widely used in the laying hen industry. In these studies, increased mortality due to FLHS was found at temperature extremes during hot weather (Couch, 1956; Greuel and Hartfiel, 1968; Ivy and Nesheim, 1973; Pearson and Butler, 1978a; Schexnailder and Griffith, 1973). In two trials, Griffith and Schexnailder (1970) found a substantial increase in the fat content of livers from hens housed in cages, during the summer months. According to these investigators the energy requirement of a hen, already lowered by the restriction of exercise in the cage, was further reduced by a high environmental temperature which discourages movement of birds and reduces heat loss (Meijering, 1979).

Temperature is a factor that is related to type of housing, and apparently contributes to the development of FLHS. Interestingly, Wolford (1971) observed in his experiments that the liver fat content in birds housed at 17°C for 28 days was significantly lower than in hens housed at 26.7°C for the same period. Lee et al. (1975) however, did not see any effect of changing the environmental temperature from 22.2°C to 30.6°C or from 30.6°C to 22.2°C on total liver fat or liver wet weight. There is some information suggesting that daily fluctuations in temperature, perhaps affected by the season of the year, stimulates hens to over consume feed. Jensen et al. (1976a) observed more FLHS in warmer vs. cooler regions of Georgia, despite feed intake being reduced at high temperatures and increased in cold temperatures. The relationship between feed intake and hepatic lipid accumulation in laying hens is not straight forward, and is likely modulated by the rate of egg production and feed composition (Leeson, 2012).
The observations in the first study of this thesis, and current observations, are partly in agreement with previous investigators. One area of agreement is that housing system influences the prevalence of FLHS. As shown in the first study hens kept in cages exhibit a higher incidence of FLHS than those kept on the floor (barn and free-range system) in flocks with a similar strain of bird, diet and other husbandry practices. As regards the effect of temperature on the prevalence of FLHS, the first study and this study showed that hens kept in battery cages in an environmentally controlled temperature (23±0.5°C) exhibit FLHS with the same frequency as hens kept in naturally ventilated sheds. As previously shown, keeping temperature controlled within the thermo-neutral zone does not decrease the incidence of FLHS (Lee et al., 1975). Other factors could have been involved. Most investigators agree that lack of exercise is a bigger contributor to FLHS. More specifically, this is related to the excess of energy produced and not spent, i.e. birds kept in battery cages have lower energy requirements. In addition, birds kept in cages at a thermo-neutral temperature require less energy, as they do not use the extra energy to activate mechanisms for maintaining body temperature. It is known that the body of an animal spends an important fraction of its energy to maintain core body temperature (Silva, 2006). It is thought that exposure to cold or heat induces stress and influences lipid metabolism in the fowl. Jaussi et al. (1962) demonstrated the role of both low- and high-temperatures as stress factors on the incidence of increased hepatic lipid deposition and FLHS, by injecting adrenocorticotrophic hormone (ACTH) and producing a similar response in treated chickens.

To sum up, hens under the influence of multiple factors such as restricted movement in cages (associated with an increase in BW), increased egg production (including increased levels of oestradiol and lipid metabolites, such as TG) nutritional factors and other stress factors (including temperature changes) are predisposed to FLHS. Low, thermo-neutral or high temperatures may contribute in different physio-pathological ways to increased hepatic lipid deposition and BW. In the current epidemiological study, heavier birds (the average BW of dead birds was 2008±107 g) in a flock were more likely to have the condition than the lighter birds. The greater BW reflects the effect of factors mentioned above including the lack of activity of caged birds. Liver weights were measured in this study, however due to the fact that some hens had large blood clots in the abdominal cavity and in some other birds the haemorrhage was subcapsular or in the form of big haematomas inside the liver, there were big discrepancies on liver weights, with livers weighing from 20 g to 85 g (data not shown).

In this study, it was expected that blood parameters would be sufficient to identify anaemia caused by haemorrhage or haematoma of the liver of hens. Many investigators recommend measurements
of plasma enzymes to test for FLHS (Diaz et al., 1999; Pearson et al., 1978b; Walzem et al., 1993) and suggest that enzyme activities are indicative of liver damage in birds, in particular AST, LDH, and GDH. In this study, GGT was measured as it is a more sensitive and specific indicator of liver dysfunction in mammals and birds (Center, 2007; Lim et al., 2007). From studies in humans and animals it has been shown that there is a correlation between GGT and TG in patients with steatohepatitis (Lim et al., 2007; Sakugawa et al., 2004). In this study, GGT was significantly elevated on 2 occasions at 35 wk (birds in Farm 3 flock 2) and at 68 wk of age (birds in Farm 2). In both cases this increase correlated ($P<0.05$) with increased TG levels.

Circulating levels of metabolites (CHOL, TG, and GLU) and TP are seen as biomarkers of energy or nutritional status and metabolic disorders (Bedogni et al., 2006; Brown et al., 2010). Circulating levels of TG were significantly increased in hens surveyed in this study, and were associated with increased egg production at this period of laying cycle. It is recognised that laying hens normally have increased circulating lipid metabolites in particular TG from 2-10 fold during egg production (Klasing, 1998), and develop hepatic steatosis to meet the requirements for yolk lipid synthesis and deposition (Hansen and Walzem, 1993). Data recorded in this survey demonstrated that very high levels of TG were measured in hens from 35 to 60 wk of age. In some cases this increase was associated with a slight rise in GLU concentrations. It appears that the diet and level of egg production influenced plasma metabolite levels. At 68 wk, birds in Farm 2 (fed a corn-based diet) had high concentrations of TG and GLU demonstrating that these birds had excess energy intake (at least more energy that they needed for egg production at this stage). It was not possible to follow the fate of flocks or birds sampled in this study, and determine if these birds could have been later prone to FLHS.

It is pertinent to remember that death from FLHS occurs only in extreme cases following massive liver haemorrhage; therefore, it is likely that a significant number of hens within the flock were also suffering from “sub-acute and chronic FLHS”. This may have caused a drop in egg production, but little or no change in mortality. However, such hens may exhibit reproductive dysfunction (Chen et al., 2006). This is likely the result of chronic liver damage and impairment of the transport of triglycerides, phospholipids, and cholesterol from the liver to the ovary (Walzem et al., 1999), resulting in decreased yolk formation and egg production. This could have been the case with birds in Farm 1, where there was a drop in %HDP in hens from 46 to 54 wk of age, while the mortality rate was constant.
The results of this study demonstrated that the acute and chronic forms of FLHS are a significant source of losses in egg production for cage egg producers. It was assumed that on-farm management factors such as housing and different nutritional conditions could have contributed to excessive liver fat deposition and increased BW, presumably resulting in greater flock deaths due to FLHS. Monitoring flocks for increased BW and occurrence of FLHS (through systematic post-mortem examinations of dead hens) should be a priority and may assist in preventing FLHS. Furthermore, the development of a rapid, non-invasive technique (e.g. a body uniformity index for laying pullets) could help cage egg producers to identify birds predisposed to FLHS to the onset of egg laying and make important management decisions for their flocks while maximizing egg production and hen welfare.
CHAPTER 6
EXPERIMENTAL INDUCTION OF FLHS

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6.1 Introduction

In the preceding Chapters, it was shown that the housing system plays a major role in the occurrence of FLHS in laying hens. The occurrence of FLHS was found to be high in commercial cage layer operations ranging from 40 to 70% of dead necropsied birds. Recent USA reports (Mete et al., 2013; Trott et al., 2013), indicate that FLHS was the most common non-infectious cause of mortality in backyard laying hens. As previously demonstrated by other investigators and
discussed in Chapter 2, there are many factors involved in the pathogenesis of FLHS including nutrition, which seems to be a major predisposing factor in backyard hens.

Despite FLHS causing significant economic losses, it is often overlooked and neglected by producers due to difficulties in detecting and preventing this metabolic disorder (Decuypere and Verstegen, 1999; Julian, 2005; Scheele, 1997). In Chapters 4 and 5 it was emphasised that post-mortem of dead hens is the only way to monitor the presence of the FLHS in a flock, while BW records and blood tests could help to evaluate and track the risk of the condition developing in a commercial laying flock. However, a greater understanding of the pathogenesis and methods to detect the condition are required if the risk of FLHS developing in a flock is going to be reduced.

The study of FLHS, as it occurs in flocks in the field (i.e. the initiation, progression and resolution of this disease), is very difficult as it occurs sporadically and over an extended period of time. An experimental model could assist greatly in investigating this disease and models have been attempted in the past as discussed below and in Chapter 2. Many of these models relied on manipulating the dietary energy (output/input) ratio, or elevation of circulating oestrogen levels, for a successful induction of FLHS.

Fatty liver haemorrhagic syndrome has been induced in immature female and male chickens (Polin and Wolford, 1977) and mature laying hens (Stake et al., 1981). Polin and Wolford (1977) either force-fed alone (amount of feed equal to 125% and 150% of ad libitum intake) or combined with hormonal treatments (three times a week for 21 days) in an attempt to induce FLHS in immature chickens. Force-feeding produced a gradient response in hepatic steatosis (as measured by percentage of fat in the liver, and the ratio of fat/fat-free liver dry weight), but did not reproduce FLHS. In contrast, intramuscular (i.m.) injections of β-estradiol-17-dipropionate (E2) at 2 mg/kg BW, three times a week for 21 days, produced a gradient response in haemorrhagic score, and an increase in ad libitum feed intake, with no significant differences between sex or breed (Polin and Wolford, 1977). However, females of both breeds accumulated significantly more liver fat than males.

Stake et al. (1981) used an endocrine (E2) model to study breed difference susceptibility to FLHS. Exogenous E2 was administered i.m. every 4 or 5 days (5.0 or 7.5 mg E2/kg BW) to induce FLHS in 9-month-old Rhode Island Red (RIR) and White Leghorn (WL) hens. The RIR hens exhibited ataxia and opisthotonus; 30% died from hepatic haemorrhage within 14 days. No WL birds (that were similarly treated for 32 days) died or showed neurologic disorder, thereby indicating major
breed differences in response to exogenous E₂. There were increases in liver lipid percentage, liver haemorrhage, and plasma volume due to E₂ injections. Other reports have shown that exogenous E₂ can also induce fatty liver in immature birds (Balnave, 1971; Chawak et al., 1997; Harms et al., 1977; Klimis-Tavantzis et al., 1983; Pearson and Butler, 1978b).

It appears from these studies that FLHS is more likely to be induced when mature laying hens are used. Moreover, as FLHS is usually observed in the latter half of egg production, it was decided to use laying hens after the onset of lay and before the peak of production. It is recognized that FLHS is commonly observed in high egg-producing birds, therefore the administration of exogenous oestrogen in the laying hen that has already started the production cycle should further stimulate lipid synthesis, and cause increased hepatic fat deposition. Moreover, a number of investigators administered oestrogen while feeding hens ad libitum or in combination with force feeding, to create a positive energy balance, which was seen as a necessity for FLHS to occur (Harms et al., 1977; Pearson and Butler, 1978b; Polin and Wolford, 1977; Stake et al., 1981).

In this thesis, the amount of the feed consumed by hens was seen as a factor that might be manipulated to either; enhance the incidence of FLHS (if given ad libitum), or reduce lipid synthesis and the incidence of the FLHS (if controlled or restricted). Therefore, the aims of this study were to:

(i) Induce FLHS in laying hens by using exogenous E₂;
(ii) Manipulate the feed intake of the hens, and evaluate the effect of feed intake in the development of FLHS.

6.2 Materials and methods

6.2.1 Laying hens and housing conditions

Experiments were carried out at the Gatton Layer Facility, University of Queensland. One hundred and eight 30-week-old Hy-Line Brown laying hens were housed individually in stainless steel cages and kept in a controlled environment shed. The temperature of the shed ranged from 22°C to 24°C, and hens were exposed to a photoperiod of 16 hours of light and 8 hours of dark. Birds were fed a commercial layer (wheat-sorghum-soybean) diet that contained CP - 17.5%; ME - 11.5 MJ/kg; Ca - 4.1%; Available P - 0.40%; Na - 0.18%; Lysine - 0.85%, Methionine + Cysteine 0.77% (Appendix 2).
6.2.2 Experimental design: induction of FLHS in the laying hen

In this study, the experimental protocol was based on the model proposed by Stake et al. (1981) to induce FLHS by injecting exogenous E2. Oestradiol (5mg/kg BW in 0.5ml corn oil) was injected every 4th day for 20 days (6 injections in total). The dose was delivered i.m. into the pectoral muscle and adjusted to initial BW. In addition to exogenous E2 administration, energy intake (i.e. total feed intake) of birds was manipulated to investigate the effect of ad libitum vs. restricted feed intake on the occurrence of FLHS. Hens in the feed-restriction treatment were given 10% less feed than the breeder’s recommendation (HY-Line, 2008). It was anticipated that this level of restriction (10%) would reduce the amount of excessive energy (fat synthesis and deposition) in birds without disturbing the physiology of egg production and body weight maintenance.

Hens were randomly allocated into six groups (18 hens each group), two untreated (one group had restricted feed intake), two groups injected with the E2 (one group had restricted feed intake), and two groups treated with oil only (one group had restricted feed intake). The treatment program and sampling was performed as indicated in Table 6.1. At each sampling point, three hens per treatment were necropsied for liver macroscopic and microscopic evaluation.

6.2.3 Blood tests

Blood samples were taken from the wing vein using individual EDTA vacutainers and individual blood tubes. At each sampling point, 6 birds per treatment were bled. Whole blood was used to measure haematological parameters in an automated analyser (CELL-DYN® System 3700CS, Abbott Park, IL 60064), including the total number of red blood cells (RBC), haematocrit (HCT) and haemoglobin (HGB) concentrations, and the total number of white blood cells (WBC) and white blood cell count differentials (percentages).

For other analyses, blood was centrifuged (1,500 rpm for 10 min) and plasma was stored at -20ºC. Blood concentrations for plasma metabolites were determined using commercial kits and a chemistry system (VetTest chemistry analyser, IDEXX Laboratories, Inc. USA). For detailed information on metabolite (CHOL, TRG, TP, and AST) testing see Chapter 3.3.2. In addition to metabolites, measurements of plasma fibrinogen levels were carried out. Plasma fibrinogen content was determined by the refractometer method (for details see Chapter 3.3.2).
Serum E$_2$ concentrations were measured by radio-immunoassay (RIA), using coated tube technology (Spectria) from Orion Diagnostics as described in Chapter 3.3.1.

Table 6.1  Summary of the treatments and hen sampling.

<table>
<thead>
<tr>
<th>Groups$^1$</th>
<th>Treatment/day</th>
<th>Dose/form/route</th>
<th>Sample/time$^2$</th>
<th>Feed intake$^3$</th>
<th>Performance records$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAL</td>
<td>E2</td>
<td>5mg/0.5mL oil/kg BW, i.m.</td>
<td>Blood, Liver; 0h, 24h, 1wk, 2wk, 3wk, 4wk</td>
<td>Ad libitum</td>
<td>HDP, FC</td>
</tr>
<tr>
<td></td>
<td>Day 1, 5, 10, 15, 21.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERF</td>
<td>E2</td>
<td>5mg/0.5mL oil/kg BW, i.m.</td>
<td>The same</td>
<td>Restricted</td>
<td>The same</td>
</tr>
<tr>
<td></td>
<td>Day 1, 5, 10, 15, 21.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OAL</td>
<td>Oil</td>
<td>0mg/0.5mL oil/kg BW, i.m.</td>
<td>The same</td>
<td>Ad libitum</td>
<td>The same</td>
</tr>
<tr>
<td></td>
<td>Day 1, 5, 10, 15, 21.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF</td>
<td>Oil</td>
<td>0mg/0.5mL oil/kg BW, i.m.</td>
<td>The same</td>
<td>Restricted</td>
<td>The same</td>
</tr>
<tr>
<td></td>
<td>Day 1, 5, 10, 15, 21.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAL</td>
<td>Untreated</td>
<td>-</td>
<td>The same</td>
<td>Ad libitum</td>
<td>The same</td>
</tr>
<tr>
<td>CRF</td>
<td>Untreated</td>
<td>-</td>
<td>The same</td>
<td>Restricted</td>
<td>The same</td>
</tr>
</tbody>
</table>

$^1$EAL = oestrogen-treated (ad libitum feed intake); ERF = oestrogen-treated (restricted feed intake); OAL = oil-treated (ad libitum feed intake); ORF = oil-treated (restricted feed intake); CAL = control (ad libitum feed intake); CRF = control (restricted feed intake);

$^2$Last treatment with exogenous E$_2$ was performed on day 21, but samples and records were taken also 1 week post-treatment, on day 28.

$^3$Feed was restricted by 10% of the daily feed intake recommended by breeder for birds of this age (30 to 35 wk of age).

$^4$HDP = hen day production; FC = feed consumption; BW = body weight; i.m. = intramuscular.
6.2.4 Liver tests

Necropsies were conducted at each time point of sampling, and when hens died during the experiment. The liver was removed weighed, and individually examined for the presence of haemorrhagic lesions. Haemorrhages were counted on both dorsal and ventral surfaces of the liver. Liver haemorrhages were graded on a scale from 0 to 5 (for details see Chapter 3.3.3. and Chapter 5.3.3). Liver weight (g), and relative liver weights (liver to body weight ratios, g/100g body weight) were calculated and recorded. Liver samples were taken for the determination of total liver fat content, and histological examinations. The liver lipid content was determined by the method of Folch et al. (1957) as described in Chapter 3.3.5.

Liver histology was conducted as described in Chapter 3.3.4. For each section of liver, randomly located areas were assessed using light microscopy (40x and 100x magnification). A digital camera connected to the microscope was used to take images. The histological slides were evaluated for the presence of intact and nucleated fat cells; the presence of vacuoles (a fat vacuole was considered to be any non-staining area of cytoplasm with a sharply defined border); inflammation, as evidenced by focal infiltration with leukocytes; and the presence of haemorrhage (determined according to the dilatation of arteries and veins, including focal infiltration of liver tissue with RBC).

6.2.5 Laying hen performance

Production and mortality records were used to calculate: egg day production, expressed as percentage of eggs produced per hen per day (hen day production, %HDP), and egg weights (g); mortality rates expressed as percentages of cumulative mortality from start of the experiment; body weights (BW g). In addition feed consumption (FC) g/bird/day was calculated for birds that were given an ad libitum diet.

6.3 Statistical analysis

For this study, a null-hypothesis was established saying that treatments (including both, E₂ and restricted feed intake) will not cause any change in parameters measured in treated birds, so treated and untreated birds will have similar parameters over time. Data were collected at each sampling point, and values were subjected to two-way repeated measures ANOVA.
All analyses were performed using the General Linear Model (GLM) procedure of Statistical Analysis Software (SAS Institute Inc, 2001). For further interpretation of data, General Linear Model (GLM) procedures were used. When significant differences were found, comparisons among treatments were conducted by Duncan’s multiple comparison tests. Statements of significance were based on $P<0.05$. Correlations between parameters were determined using Pearson correlation coefficients.

6.4 Results

6.4.1 Evaluation of the FLHS model

Data on FLHS incidence (determined after the necropsy of euthanised birds, or birds that died during the experiment), mortality rate, liver haemorrhagic score and liver fat content of treated and control birds are presented in Table 6.2.

Data on mortality and liver haemorrhagic score demonstrated that all birds (100%) treated with E$_2$ developed FLHS and presented different signs at different stages of the development of the condition. Birds treated with exogenous E$_2$ and fed *ad libitum* developed advanced signs of FLHS (liver haemorrhagic score = 4-5, revealed in 87.5% of birds in this treatment/group). Three (or 18.8%) birds in this treatment died due to liver haemorrhage or haematomas. Oestrogen-treated birds in the restricted-feed intake group developed FLHS in a similar way to E$_2$-treated birds in the *ad libitum* fed group; however, the incidence of FLHS in the *ad libitum* fed group was higher ($P<0.05$; 87.5 % vs. 68.8%, respectively). The restricted feed intake group showed ca. 20% less incidence of FLHS than the *ad libitum* fed group. There were no mortalities in untreated or oil-treated groups. All control groups had a low incidence of signs FLHS, in terms of haemorrhagic score (only 6.3 and 18.8 % of birds revealed signs of small haemorrhages or petechies in the liver) with control hens in the restricted feed intake group having the lower incidence of FLHS (only 6.3%, and a liver haemorrhagic score 1).

Data on other liver measurements indicated that treatment with E$_2$ induced a significant ($P<0.05$) increase in liver weight, and liver fat content (Table 6.2). In particular the EAL-treated group showed an increase in fat content in the liver over 50%. The liver weights to BW (g/100g) ratios were significantly ($P<0.05$) increased in E$_2$-treated hens when compared to controls (2.9 vs. 2.1).
Table 6.2  Effect of oestrogen injections and feed restriction on the incidence of FLHS, mortality, liver haemorrhagic score, liver weight and fat content, and relative liver weight of laying hens.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>N of birds</th>
<th>FLHS (%)</th>
<th>Haemorrhagic score</th>
<th>Liver weight (g)</th>
<th>Fat content (%)</th>
<th>Liver weight to BW ratio (g/100g)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAL&amp;OAL&lt;sup&gt;3&lt;/sup&gt;</td>
<td>36</td>
<td>18.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1-2</td>
<td>38.6±5.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.2±2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRF&amp;ORF&lt;sup&gt;3&lt;/sup&gt;</td>
<td>36</td>
<td>6.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>39.2±4.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.8±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EAL</td>
<td>18</td>
<td>87.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4-5</td>
<td>53.0±6.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.4±5.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.8</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>12.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERF</td>
<td>18</td>
<td>68.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4-5</td>
<td>47.4±5.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.6±3.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td>12.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
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</tbody>
</table>

<sup>1</sup>Data are presented for the whole experimental period; N of birds sacrificed at each sampling point was 3 birds/treatment. At the end of the experimental period, all birds were sacrificed and underwent post-mortem examination.  
<sup>2</sup>EAL = oestrogen-treated (ad libitum feed intake); ERF = oestrogen-treated (restricted feed intake); OAL = oil-treated (ad libitum feed intake); ORF = oil-treated (restricted feed intake); CAL = control (ad libitum feed intake); CRF = control (restricted feed intake).  
<sup>3</sup>There were no significant differences between untreated and oil-treated groups; therefore, data are pooled and presented together for control untreated and oil treated groups for both ad libitum and restricted feed intake birds;  
<sup>4</sup>Data on the occurrence of FLHS are divided to reflect haemorrhagic scores (next column) and so indicate the range and severity of the lesions.  
<sup>5</sup>Values for liver measurements are presented as mean ±SD (n=18).  
<sup>a,b,c,d</sup>Values within a column with different superscripts differ significantly (P<0.05).
6.4.2 Plasma E\textsubscript{2} concentration

The baseline concentrations of E\textsubscript{2} were found to range between 350 and 500 pg/mL in all groups. In the present study plasma E\textsubscript{2} concentrations were elevated by exogenous administration of 5 mg/kg BW (Figure 6.1). The levels of E\textsubscript{2} rose rapidly in EAL and ERF treated birds and were 2 to 3 times higher than before treatment (baseline) or control birds measured at 24 h post injections (approx. 1500 and 900 pg/mL, respectively). The E\textsubscript{2} concentrations peaked twice for EAL treated birds at 1wk and 3 wk after treatments started with the highest value reached at 3 wk approx. 2115 pg/mL. The E\textsubscript{2} concentration for ERF birds was significantly lower (\(P<0.05\)) than EAL birds, however, ERF birds showed a significant increase (\(P<0.05\)) in E\textsubscript{2} concentrations when compared to E\textsubscript{2} levels of control birds.

Birds in the control group oil (ORF) had slightly lower levels of E\textsubscript{2} than other controls however this difference was not significantly different (\(P>0.05\)) from the other controls; therefore data are presented together for all controls.

EAL birds had their E\textsubscript{2} concentrations elevated over the whole period of the treatment, and the levels decreased 10 days after last treatment and reached the baseline value (Figure 6.1). It should be noted that the E\textsubscript{2} treatment did not always fit with blood sampling and E\textsubscript{2} measurements; treatments were every 4-5 days, while blood sampling was taken weekly after the first treatment.
Figure 6.1 Effects of exogenous oestradiol on plasma oestradiol concentrations in treated and control birds\textsuperscript{1,2,3}

\textsuperscript{1}EAL = oestrogen-treated (\textit{ad libitum} feed intake); ERF = oestrogen-treated (restricted feed intake); OAL = oil-treated (\textit{ad libitum} feed intake); ORF = oil-treated (restricted feed intake); CAL = control (\textit{ad libitum} feed intake); CRF = control (restricted feed intake);

\textsuperscript{2}There were no significant differences between untreated and oil-treated groups (for both diets, \textit{ad libitum}; restricted feed intake) therefore, data are pooled and presented together for control untreated and oil treated groups (CAL, OAL, CRF and ORF birds);

\textsuperscript{3}Values are presented as mean ±SD (n=6, for each time point);

Significant differences between groups are represented by *\(P<0.05\); **\(P<0.01\); ***\(P<0.001\).
6.4.3 Peripheral leukocyte counts and fibrinogen concentration

As shown in Table 6.3, at 24 hours, 1 wk, 2 wk and 3 wk post-treatment, E2-treated chickens of both groups (EAL and ERF) showed a significant \( (P<0.01) \) increase in peripheral white blood cells (WBC) or leukocytes, when compared to basal (at 0 h) levels and control hens (at 0 h and 24 h). At 2 wk post-treatments with E2, WBC counts rapidly increased \( (P<0.01) \) in E2-treated birds of EAL group and peaked with a value \( 64.8\pm4.6 \times 10^9/L \). The WBC counts stayed elevated for both E2 treated groups (EAL and ERF) in the following week (week 3) as well and were reduced at week 4 after the first treatment with E2 (or 1 week after the last treatment with E2).

Plasma fibrinogen levels were also elevated \( (P<0.01) \) over time, predominantly in the first and second week post-E2 treatment in EAL and ERF hens. Fibrinogen levels peaked earlier than the WBC counts at week 1 (the value was approx. 3 times higher than the baseline, ca. 6g/L), and continued to be elevated on week 2 in both E2 treatment groups (EAL and ERF). Thereafter, plasma fibrinogen concentrations declined and were found significantly \( (P<0.05) \) lower than the values measured before treatments (or baseline levels).

Correlation analyses showed that there was a low level of positive correlation between WBC counts with fibrinogen concentrations over time in E2 treated hens (Pearson’s correlation \( r=0.20; P=0.014 \)). The highest correlation was found at 2 wk (Pearson’s correlation \( r=0.96; P=0.001 \)). The regression line is shown in the scatter diagram (Figure 6.2) and expresses the relationship in the form of an equation which can help to predict the level of fibrinogen for a given WBC count in the case of high levels of E2.

All control hens (untreated/oil/ad libitum or restricted feed intake) had slight but insignificant \( (P>0.05) \) changes of WBC and fibrinogen levels over time.

As shown in Table 6.3 there were no significant differences \( (P>0.05) \) in RBC counts and HCT values among treated and untreated groups.
Table 6.3  Data on haematological parameters and plasma fibrinogen concentration of 
E₂ treated and control hens.

<table>
<thead>
<tr>
<th>Treatment¹</th>
<th>Time</th>
<th>WBC² (x10⁹/L)</th>
<th>RBC² (x10¹²/L)</th>
<th>HCT² (L/L)</th>
<th>Fibrinogen² g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAL</td>
<td>0 h</td>
<td>16.5ᵃ</td>
<td>2.4</td>
<td>0.30</td>
<td>2.3ᵃ</td>
</tr>
<tr>
<td>CRF</td>
<td>0 h</td>
<td>16.6ᵃ</td>
<td>2.6</td>
<td>0.29</td>
<td>2.5ᵃ</td>
</tr>
<tr>
<td>EAL</td>
<td>0 h</td>
<td>16.3ᵃ</td>
<td>2.5</td>
<td>0.30</td>
<td>2.4ᵃ</td>
</tr>
<tr>
<td>ERF</td>
<td>0 h</td>
<td>16.1ᵃ</td>
<td>2.5</td>
<td>0.28</td>
<td>2.2ᵃ</td>
</tr>
<tr>
<td>CAL</td>
<td>24 h</td>
<td>17.7ᵃ</td>
<td>2.1</td>
<td>0.26</td>
<td>2.6ᵃ</td>
</tr>
<tr>
<td>CRF</td>
<td>24 h</td>
<td>17.5ᵃ</td>
<td>2.5</td>
<td>0.27</td>
<td>2.8ᵃ</td>
</tr>
<tr>
<td>EAL</td>
<td>24 h</td>
<td>31.1ᵇ</td>
<td>2.2</td>
<td>0.26</td>
<td>4.5ᵇ</td>
</tr>
<tr>
<td>ERF</td>
<td>24 h</td>
<td>25.7ᵇ</td>
<td>2.3</td>
<td>0.27</td>
<td>4.8ᵇ</td>
</tr>
<tr>
<td>CAL</td>
<td>1 wk</td>
<td>16.9ᵃ</td>
<td>2.2</td>
<td>0.26</td>
<td>2.5ᵃ</td>
</tr>
<tr>
<td>CRF</td>
<td>1 wk</td>
<td>16.2ᵃ</td>
<td>2.5</td>
<td>0.29</td>
<td>2.6ᵃ</td>
</tr>
<tr>
<td>EAL</td>
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<td>28.4ᵇ</td>
<td>2.7</td>
<td>0.27</td>
<td>5.5ᵇ</td>
</tr>
<tr>
<td>ERF</td>
<td>1 wk</td>
<td>25.2ᵇ</td>
<td>2.3</td>
<td>0.27</td>
<td>5.8ᵇ</td>
</tr>
<tr>
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<td>2.4ᵃ</td>
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<td>16.6ᵃ</td>
<td>2.2</td>
<td>0.26</td>
<td>2.8ᵃ</td>
</tr>
<tr>
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<td>0.24</td>
<td>5.2ᵇ</td>
</tr>
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<td>2.2</td>
<td>0.24</td>
<td>4.5ᵇ</td>
</tr>
<tr>
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<td>18.0ᵃ</td>
<td>2.2</td>
<td>0.26</td>
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</tr>
<tr>
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<td>3 wk</td>
<td>17.2ᵃ</td>
<td>2.4</td>
<td>0.30</td>
<td>3.0ᵃ</td>
</tr>
<tr>
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<td>3 wk</td>
<td>55.9ᶜ</td>
<td>1.9</td>
<td>0.28</td>
<td>1.2ᵃ</td>
</tr>
<tr>
<td>ERF</td>
<td>3 wk</td>
<td>53.3ᶜ</td>
<td>2.0</td>
<td>0.25</td>
<td>1.0ᵃ</td>
</tr>
<tr>
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<td>4 wk</td>
<td>20.0ᵃ</td>
<td>2.2</td>
<td>0.29</td>
<td>2.3ᵃ</td>
</tr>
<tr>
<td>CRF</td>
<td>4 wk</td>
<td>18.2ᵃ</td>
<td>2.3</td>
<td>0.28</td>
<td>2.6ᵃ</td>
</tr>
<tr>
<td>EAL</td>
<td>4 wk</td>
<td>20.0ᵃ</td>
<td>3.0</td>
<td>0.26</td>
<td>1.7ᵃ</td>
</tr>
<tr>
<td>ERF</td>
<td>4 wk</td>
<td>20.2ᵃ</td>
<td>2.8</td>
<td>0.25</td>
<td>1.5ᵃ</td>
</tr>
</tbody>
</table>

¹EAL = oestrogen-treated (ad libitum feed intake); ERF = oestrogen-treated (restricted feed intake); OAL = oil-treated (ad libitum feed intake); ORF = oil-treated (restricted feed intake); CAL = control (ad libitum feed intake); CRF = control (restricted feed intake); WBC = white blood cells; HCT = haematocrit;
²Values are expressed as mean (n=6). Means with different superscripts (a-c) within a column are significantly different (P<0.05).
Figure 6.2  Linear regression of WBC counts with plasma fibrinogen in oestradiol treated and control birds at 2 wk after treatment started.

Each data point represents the average of 6 measurements per treatment/group;

WBC = white blood cells.
Figures 6.3, 6.4, 6.5 and 6.6 present data on plasma metabolites (CHOL, TG, TP and AST respectively) from E\textsubscript{2} treated and control hens. As previously mentioned in the case that there were no significant differences between untreated and oil-treated groups (for both diets, \textit{ad libitum} and restricted feed intake) data are pooled and presented together for control (untreated) and oil treated groups (CAL, OAL, and ORF birds).

Hens treated with E\textsubscript{2} showed increased ($P<0.01$) levels of plasma CHOL and TG at 24 h and week 1, 2, and 3 after treatments (Figure 6.3 and Figure 6.4). In both E\textsubscript{2} treated groups (EAL and ERF) plasma CHOL rose after 24 h and was elevated during the experimental period, however, \textit{ad libitum} treated hens had higher ($P<0.05$) levels of CHOL than hens in restricted feed intake treatment. In terms of TG, all E\textsubscript{2} treated birds had higher ($P<0.01$) plasma levels than controls, found in all measurement points after first treatment with E\textsubscript{2}. In all control birds there were no significant changes in plasma CHOL or TG levels in all measurement points.

Measurements of plasma TP showed slight non-significant changes of TP concentrations in all birds. There were found a lower TP concentration in EAL and ERF groups at 1 wk after treatments with E\textsubscript{2} (Figure 6.5).

For this study, both AST and GGT were measured in all birds to study changes in plasma liver enzyme concentrations of treated or untreated birds. However, changes in GGT concentrations were lower and insignificant (data not shown). The levels of plasma AST are shown in Figure 6.6 revealing a significant decrease of AST levels in particular at 24 h, 1 wk, and 3 wk after first treatment with E\textsubscript{2} in both E\textsubscript{2} treated groups, EAL and ERF. At week 3, levels of AST were found lower than in birds not treated with E\textsubscript{2} however this decrease was not statistically significant. According to the schedule for E\textsubscript{2} treatment this sampling point was planned 1 day before the next treatment with E\textsubscript{2}. 

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Figure 6.3  Plasma cholesterol (mmol/L) levels in oestradiol treated and untreated birds.
Each data point is average of 6 measurements.

Figure 6.4  Plasma triglyceride (mmol/L) levels in oestradiol treated and untreated birds
Each data point is average of 6 measurements.
TG = Triglycerides.
**Figure 6.5** Plasma total protein (g/L) levels in E₂ treated and untreated birds.

Each data point is average of 6 measurements;  
TP = Total protein.

**Figure 6.6** Plasma AST (IU/L) levels in E₂ treated and untreated birds.

Each data point is average of 6 measurements;  
AST = Aspartate aminotransferase.
6.4.5 Liver macroscopic and histological examination

The liver tissue from birds euthanised at sampling points, or birds that were euthanised or found dead (during experimental period) were collected and examined. The majority of livers were pale, swollen and friable with different grades of haemorrhages and haematomas on both surfaces (dorsal and ventral) or in the edges of both lobes. In advanced cases (haemorrhage score 4 or 5), liver tissue was ruptured and small superficial or deep haematomas and or large blood coagula were found inside the abdominal cavity (Figure 6.7a,b and 6.8a,b). Hens with liver rupture died during the experiment; most deaths in E\textsubscript{2}-treated birds happened in the first or second week of treatment (see Table 6.2 for the mortality rates). Hens that had haematomas survived and were diagnosed with FLHS at necropsy after euthanasia (Figure 6.8). Data on liver tests (i.e. relative weights and fat content) showed that E\textsubscript{2} treatment resulted in a significant ($P<0.05$) increase in liver relative weight and fat content of treated hens vs. controls (Table 6.2).

Oestradiol treatment resulted in an increased infiltration of liver tissue and hepatocytes with fat. This can be seen macroscopically in Figure 6.7. Histologically, all livers had significant slight or moderate lipid accumulation (Figure 6.9 a, b). However, E\textsubscript{2}-treated birds demonstrated severe fat deposition, as indicated by the presence of small or large fat droplets in hepatocytes, or large vacuoles of fat filling the cytoplasm and distending hepatocytes (Figure 6.10 a, b). In addition to fat deposition, histological sections of E\textsubscript{2}-treated birds indicated focal inflammatory (heterophilic and/or lymphocytic/ mononuclear) infiltration, local haemorrhage and congestion of sinusoids, demonstrating an increased incidence of inflammation and haemorrhage Fig 6.11 a, b). Massive lipid infiltration, diffuse inflammatory infiltration and congestion were observed especially in the liver parenchyma of birds that macroscopically demonstrated severe lesions of FLHS.
Figure 6.7  Livers from oestradiol treated hens showing increased fat content, and haematomas and haemorrhage.

Pictures presented here show livers removed from birds after first week of treatment with E$_2$, showing an increased fat content (fat shown on the surface of the organ) and small haematomas (a) and haemorrhage or blood coagula in the abdominal cavity (b).
Figure 6.8  Livers from oestradiol treated hens showing superficial and deep haematoma.

Pictures presented here show livers removed from birds at the end of the experiment (week 4) showing a superficial (a) and a deep haematoma (b). It was suggested that these birds survived until experiment ended but stopped egg production in week 2.
Figure 6.9 Histological sections of livers from hens in the control group. Pictures showing normal structure of the tissue with some fat deposition (yellow arrows) in hepatocytes, and small fat vacuoles around the nucleus (microvesicular fatty change); H&E stain, using x200 (a) and x400 (b) magnification; portal triad = PT.
Figure 6.10  Histological sections of livers from oestradiol treated hens (after week 1). Yellow arrows showing fat vacuoles in hepatocytes (a & b) and fat droplets in the enlarged sinusoid (a); nucleus disappeared or displaced to periphery in hepatocytes (macrovesicular steatosis). Note focal infiltration with leukocytes (green arrow) near central vein (CV); H&E stain, using x400 magnification.
Figure 6.11  Histological sections from livers of oestradiol treated hens (after week 2).

Extensive tissue infiltration with fat droplets (yellow arrow), leukocytes (green arrow), and erythrocytes (red arrow); the infiltration is most prominent near the central vein (a) and extends outward toward the portal tract. Hepatocytes are paler (a) and bigger (b) due to macrovesiculas or vacuolation; H&E stain, at x400 magnification.
6.4.6 Body Weight

Body weights of birds in both *ad libitum* fed groups (CAL and EAL birds) increased after the first week of the experiment. However, the difference was not statistically significant (Figure 6.12). Hens in both restricted feed intake groups (CRF and ERF) had their BW reduced (*P*<0.01) in the second week of the experiment or when compared to their initial body weight or hens fed *ad libitum*. In the second week of treatments, BW of CRF and ERF birds continued to remain lower (without recovering), and continued to slightly reduce until the last week of the experiment (at week 4, i.e. 1 wk after the treatment was interrupted). At week 4, hens in restricted feed intake groups had lower BW (*P*<0.01) than all other hens (CAL vs. CRF, 1892 vs. 1687g). At week 4 there was a slight increase in BW of CAL and EAL hens as compared to their initial BW, however this was statistically not significant (*P*=0.06).

![Figure 6.12](image_url)  
**Figure 6.12** Body weights (g) of treated and control birds\(^1,2\)

\(^1\)CAL = control and *ad libitum*; CRF = control and feed restricted; EAL = oestrogen-treated and *ad libitum*; ERF = oestrogen-treated and feed restricted;  
\(^2\)Values are presented as mean ±SEM (initial number was n=16, thereafter the number was reduced in all groups as every week 3 hens per group were euthanised). There were no significant differences between untreated and oil-treated groups; therefore, data are pooled and presented together. Significant differences between groups are represented by *P*<0.05.
6.4.7 Egg production

As shown in Figure 6.13, hens had achieved their peak of production at 30-31 wk of age, and entered the experiment with the majority producing over 95%. Egg production was not affected after the first week of experiment, although the calculations of %HDP showed that there was a slight decrease ($P>0.05$) in the number (and %) of the eggs produced in the first week from all hens, untreated and treated hens. Hens in both restricted feed intake groups had a slight decrease in egg production at wk 1 and wk 2; however, only at week 3 and 4 did the %HDP decreased significantly ($P<0.05$) in CRF and ERF hens when compared to *ad libitum* fed hens. One week after the treatment was interrupted (at week 4), all hens treated with E$_2$ (in the *ad libitum* and restricted feed intake diet groups) and untreated hens (in the restricted feed intake diet group) had reduced ($P<0.05$) %HDP when compared to initial %HDP at 30wk of age or hens in the *ad libitum* control group.

![Egg production graph](image)

**Figure 6.13** Egg production (HDP) of treated and control birds$^{1,2}$

$^1$CAL = control (*ad libitum* feed intake); CRF = control (restricted feed intake); EAL = oestrogen-treated (*ad libitum* feed intake); ERF = oestrogen-treated (restricted feed intake); HDP=Hen day production.

$^2$Values are presented as mean ±SEM (initial number was n=16, thereafter the number was reduced in all groups as every week 3 hens per group were euthanised). There were no significant differences between untreated and oil-treated groups; therefore, data are pooled and presented together. Significant differences between groups are represented by *$P<0.05$.*
6.4.8 Egg weight

Egg weight data of control and treated hens are shown in Figure 6.14. Hens entered the experiment with an average of egg weight of 57.8 g. There were slight changes (in both directions) in egg weights of hens after first and second week of the experiment; however none of differences was statistically significant. At Week 3 post-initial treatment with exogenous E₂, egg weights of E₂-treated ad libitum diet hens were increased significantly \((P<0.05)\) (Figure 6.14). At scheduled necropsy hens from EAL group had their ovaries full with large yolks (Figure 6.15) and their livers were very fatty and fragile. Egg weights from hens in the restricted feed regimen were slightly decreased during the experiment; there was a significant decrease in egg weights of these hens at Week 4 of the experiment.

Correlation analyses between E₂ levels and egg weights of E₂ treated hens at wk 3 showed a high positive correlation (Pearson’s correlation, \(r=0.90; P<0.01\)).

![Figure 6.14](image)

**Figure 6.14** Effects of exogenous oestradiol on egg weights\(^1\) of treated and untreated birds.

\(^1\)CAL = control (ad libitum feed intake); CRF = control (restricted feed intake); EAL = oestrogen-treated (ad libitum feed intake); ERF = oestrogen-treated (restricted feed intake); \(^2\)Values are presented as mean ±SEM (initial number was \(n=16\), thereafter the number was reduced in all groups as every week 3 hens per group were euthanised). There were no significant differences between untreated and oil-treated groups; therefore, data are pooled and presented together. Significant differences between groups are represented by \(*P<0.05\)
Figure 6.15  Yolks, an egg and liver from hens treated with oestradiol (EAL group).
EAL = oestrogen-treated (*ad libitum* feed intake) group.
These samples were taken at week 2 post-treatment in hens that were randomly euthanised for sampling.

6.4.9  Feed intake of hens fed *ad libitum*

There were no significant differences in the feed intake of hens in both groups fed *ad libitum* (E2 treated or control hens). However, hens in CAL, EAL consumed on average 115 to 122 g/bird/day during the whole experimental period.
6.4.10 Ovary

The ovary of hens was inspected for internal ovulations, internal oviposition, ovarian enlargement or regression and follicular atresia. There was an enlargement of the ova in EAL treated hens (Figure 6.16) compared to control hens, while hens in CRF and ERF showed a reduction of large yellow follicles. No internal ovulations were found in any of treated or untreated birds.

**Figure 6.16** The ovary of a laying hen showing the stroma (pool of undifferentiated follicles) and large yellow follicles.

From a hen in the control group (a), and a hen from oestrogen-treated (*ad libitum* feed intake) group (b).
6.5 Discussion

The laying hen model described by Stake et al. (1981) was employed in this study. It successfully induced FLHS under experimental conditions using modern laying hen genotypes. Stake et al. (1981) had shown significant breed differences in response to exogenous E₂ injections with the response of White Leghorn hens mimicking clinical cases. In the model, two dosing regimens were used and in the current study, the lower oestrogen dose (5mg/kg BW) was applied and FLHS was induced in hens. In the adult bird, the rate of secretion of oestradiol by the ovary is of the order of 1–2.0 mg oestradiol/24 h (Hawkins et al., 1969), while the clearance rate for oestradiol has been calculated to be between 6.3 and 11 ng/min or 9 and 16 μg/day (Johnson and van Tienhoven, 1981; Tsang and Grunder, 1984). The current results demonstrate that a dose 5mg/kg BW of E₂ (which is equivalent to 1.25 mg/kg BW/day) increased circulating concentrations of the hormone sufficiently above physiological values to induce the condition without the side effects noted by Stake et al. (1981) in Rhode Island Red hens.

The successful application of the model allowed further investigation of the role of oestradiol in the condition. Many investigators had previously suggested that oestrogen, along with a positive energy balance, is necessary for the production of FLHS (Haghighi-Rad and Polin, 1982a; Harms et al., 1977; Pearson and Butler, 1978b; Polin and Wolford, 1977; Stake et al., 1981). The model also allowed the effect of feed intake (ad libitum vs. restricted feed intake) on the incidence and progression of FLHS to be studied.

6.5.1 Oestradiol

The disease was experimentally induced in laying hens of 30 weeks of age after injections of hormone oestradiol. The administration of exogenous E₂ was associated with significant changes in the liver relative weights, and macroscopic (fat depositions, haemorrhages and haematomas) and microscopic (liver structure) alterations. The reproduction of the disease in the hen was associated with an increased mortality due to FLHS outbreak.

The measurements of E₂ concentrations demonstrated that E₂ levels were significantly elevated (P<0.01) in treated hens, and were responsible for all significant differences in recorded parameters. In laying hens, baseline E₂ increases 1 to 2 days after photostimulation and is maintained at a high level until egg production starts to decline (Etches and Cheng, 1981). At the same time as E₂ elevation, hepatic lipogenesis is increased dramatically in hens in order to meet
the demand for vitellogenesis (Hansen and Walzem, 1993). Although the main products of de novo hepatic lipogenesis are triglycerides, the liver is also the major site of cholesterol and phospholipid synthesis (Chapter 2). These lipids, along with protein, are the main components of lipoproteins. In the laying hen, many factors, both external (e.g. nutritional and environmental factors) and internal (e.g. hormones and other mediators), may stimulate lipid metabolism and increase fat deposition in the liver and abdominal cavity. The increase in liver fat content is a characteristic of hens, and is related to special structural and physiological features participating in lipid absorption, synthesis, distribution and transport in to intra- and extrahepatic compartments, including the ovary (see Chapter 2). As emphasized in the literature review, dietary lipids are absorbed and secreted directly into the portal blood system, in the form of VLDL and are termed portomicrons. Lipogenesis that occurs mainly in the liver under the influence of E2 is particularly active in females producing eggs. Exogenous and endogenous lipids once secreted into the blood are transported by the blood to the ovary as components of lipoproteins. Fatty livers are normal for egg producing hens and occur when the increase in lipogenesis exceeds the capacity of synthesis and secretion of lipoproteins (Hermier, 1997).

In this study, E2 level rose significantly in birds exposed to exogenous oestrogen, and peaked some 3 to 4 times higher than untreated birds. Under the influence of E2, hens fed ad libitum experienced severe FLHS which was associated with an increase in mortality (approx. 20% of treated hens in this group vs. 0% of control ad libitum fed hens). Hens that did not die from FLHS were diagnosed with FLHS at necropsy and with ca. 88% of them having very high haemorrhagic score of 4 to 5. Oestrogen-induced hens from the restricted feed group also developed FLHS, but with a lower frequency in mortality (ca. 6.5% vs. 20%, for restricted feed vs. ad libitum hens, respectively) and haemorrhagic score of 4 to 5 (69% vs. 88% for restricted feed vs. ad libitum hens, respectively).

6.5.2 Feed intake

As expected, ad libitum fed hens demonstrated a higher incidence of FLHS than hens with a restricted feed intake. This was in agreement with previous studies (Haghighi-Rad and Polin, 1982a; Harms et al., 1977; Lee et al., 1975; Pearson and Butler, 1978b; Stake et al., 1981) that birds with a greater feed intake are more at risk from FLHS than birds with a lower feed intake. Birds in control untreated groups did not develop FLHS and showed low haemorrhagic scores at necropsy.
In response to restricted feed intake, birds in CRF and ERF groups demonstrated a decline in BW at week 2, 3, and 4 after the restricted feed intake diet started. These birds also had a change in egg production (%HDP), which was significantly reduced in weeks 3 and 4 of the restricted feed intake regimen. With regard to egg weights, a significant decrease ($P<0.05$) was only seen in the CRF group at week 4, while egg weights of E$_2$ treated *ad libitum* hens were significantly increased ($P<0.05$) in week 3. Reductions in BW and egg production have been previously described in hens given a restricted feed intake in experiments with fatty liver (Pearce, 1980; Sallmann and Schole, 1973; Stake et al., 1981). Pearce (1980) demonstrated significant reductions in liver total lipid content compared with *ad libitum* fed control birds and also reductions of hepatic lipogenic enzyme activities. In the current study, only changes in relative liver weight and liver fat content were measured, and they were significantly increased in E$_2$ treated hens (both *ad libitum* and restricted feed intake groups) when compared to untreated birds. Control (untreated) birds, both *ad libitum* and restricted feed intake groups, had insignificant differences in liver weights and liver fat content (Table 6.2).

Overall, restriction of feed intake reduced the occurrence of FLHS of hens treated with E$_2$, in terms of mortality and haemorrhagic score, and also affected their performance. The idea of reducing feed intake by 10% of hens treated with E$_2$ was to reduce the total energy intake or minimise the positive energy balance in hens without disturbing their physiology. In relationship to decreases in BW and egg production, it should be noted that hens fed *ad libitum* consumed ca. 120 g/bird/day, suggesting that the feed intake of hens in CRF and ERF groups was reduced more than 10% or ca. 20 g/bird/day. Perhaps this is why hens in these groups had reduced BW, egg production and egg weight at weeks 3 and 4 after the treatments started.

### 6.5.3 Hepatic responses

Injections of E$_2$ induced a significant increase in relative liver weight and liver fat content (Table 6.2), presumably through the hormonal induction of hepatic lipogenesis. The histological examination of liver samples showed that all hens had some degree of hepatic fat infiltration (microvesicular fatty change). However, hens treated with E$_2$ had consistent and severe infiltrations with fat and fat vacuolisations (macrovesicular fatty degeneration or steatosis). This is one of the most striking early events that had been observed after E$_2$ exposure in the chicken. The current study was in accord with previous observations and demonstrated transient, massive accumulation of lipids (Figure 6.10b) in the cytoplasm of hepatocytes (Cherian and Goeger, 2004; Pearson and
Butler, 1978c; Walzem et al., 1993) in which a single, bulky fat vacuole distends the hepatocyte and pushes the nucleus and cytoplasm to the side.

Interestingly, apart from large vacuoles in the liver parenchyma, histological examination revealed fat droplets in the liver sinusoids (Figure 6.10b). Sinusoids represent distensible vascular channels that carry mixed (from portal vein and hepatic artery) blood from the edges of the lobule to the central vein. The central vein empties into the hepatic vein, then blood is delivered to the inferior vena cava (Figure 6.17a) (Sturkie, 1986). The sinusoidal wall is formed by 4 different cells: fenestrated endothelial cells, Kupffer cells (liver-resident macrophages), fat-storing cells (Ito or stellate cells or lipocytes or Vitamin A storing) and pit cells (the natural killer cells of the liver) (Figure 6.17b). Sinusoidal endothelial cells permit hepatocytes to access nutrients and macromolecules in plasma, and are also responsible for endocytosis of molecules and particles such as lipoproteins (Tanaka et al., 2011; Wisse et al., 1996). Previous investigators have not demonstrated the presence of fat droplets in the sinusoids or blood vessels of the liver in the chicken.

It is recognised that the absorption of exogenous fat in birds occurs directly via the portal blood system, as VLDL or portomicrons, which are rapidly taken up by the liver. Unlike mammals, the liver in birds is directly exposed to dietary fat, and is also the major site of lipogenesis (Bickerstaffe and Annison, 1970). One interesting characteristic of chicken liver structure is that the fenestra in the sinusoidal endothelium are smaller in size and lower, in number; therefore the endothelium is less porous, than in mammals. It has been suggested that this property prevents the hepatocytes from being swamped by dietary fat, but makes the chicken susceptible to diet-induced atherosclerosis (Fraser et al., 1986). As in women, oestrogen reduces atherosclerosis in chickens, mainly in the coronary arteries (Beaufrère, 2013; Petzinger and Bauer, 2013). The fenestrated endothelial cells act as a liver sieve by allowing macromolecules and chylomicrons to traverse through their fenestrations (sieve plates) to hepatocytes. Apparently, large particles that cannot pass the fenestrae in the sinusoidal capillary bed and cannot be also metabolized by the liver (Figure 6.9b) are trapped inside the sinusoids. These specific features of chicken liver structure will be discussed further in Chapter 7 in relation to a hen’s predisposition to fat deposition and FLHS.
Figure 6.17  Schematic drawing of blood circulation in the liver and liver sinusoidal cells.

(a) Source: http://www.siumed.edu/~dking2/erg/liver.htm;
(b) Tanaka et al. (2011).
6.5.4 Haematology

In this study haematological parameters were used to follow the progression of FLHS in the hen model. It was thought that these parameters (particularly the RBC and HCT) would help to predict any recent haematoma or haemorrhage in the liver or abdominal cavity of hens treated with E$_2$. It is recognised that the HCT is one of the most precise methods of determining loss of blood or the degree of anaemia, while the RBC count is usually evaluated with HCT to confirm the presence of anaemia and differentiate it from excessive hydration. The HCT and RBC counts are usually decreased in haemorrhages and haematomas (Lumeij, 2008). In this study, there were no significant changes ($P<0.05$) in HCT and RBC values. This could have happened because birds usually have good tolerance of the loss of up to 10% of their circulating blood volume (Sturkie, 1986). Like in other animals and humans, when birds lose a quantity of blood (under 10% of their circulating blood volume) they experience a physiological stress, yet the RBC counts and HCT generally remained only slightly depressed (Kovach et al., 1969).

In the case of small internal haemorrhages and haematomas, a simultaneous release of RBC from reserves in the bone marrow, and rapid fluid replacement or relocation across compartments happens (Sturkie and Griminger, 1986). Even though many hens had haemorrhages and haematomas, this situation was not clinically exhibited in the blood parameters measured, as several homeostatic pathways may have compensated for the amount of blood lost in haematomas. In the case of liver capsule rupture and large haemorrhages in the abdominal cavity, it has been suggested that hens die suddenly (Julian, 2005) due to hypovolemic shock (within minutes) or signs of peritonitis (within 24 h). In addition to RBC, total WBC counts were recorded to monitor the general health and the immune response of hens.

In this study, alteration of WBC counts and fibrinogen levels were found in hens treated with E$_2$ when compared to controls (Table 6.3). This is the first demonstration of increased WBC counts and fibrinogen in oestrogenised birds. As in mammals, the elevation of these parameters in birds demonstrates increased systemic inflammation and tissue repair (Latimer and Rakich, 2007). Hens treated with E$_2$ had a significant increase in leukocytes 24 h post-treatments and this number continued to increase in week 1, 2 and 3. Simultaneously, there was an elevation of plasma fibrinogen which correlated to the WBC increase, with the exception that on week 3 fibrinogen levels decreased presumably due to liver damage impacting on fibrinogen synthesis in the hepatocytes (Brunt, 2010; Nagata et al., 2007). Fibrinogen is a moderate acute phase protein that circulates in the plasma at a concentration of 2 g/L to 4 g/L with a half-life of approximately 4
days, increasing in concentration in response to most forms of tissue injury, infection, or inflammation (Gruys et al., 2005; Mosesson et al., 2001). The ability of fibrinogen to participate in the inflammatory response depends on its specific interaction with leukocyte cell surface adhesion receptors, integrins (Mosesson et al., 2001; Ugarova and Yakubenko, 2001). In humans, changes in markers of inflammations including leukocytes and fibrinogen are seen as a response to low-grade inflammation at local (leukocyte recruitment into the tissue) (Temelkova-Kurktschiev et al., 2002), and systemic (Haukeland et al., 2006) levels. The discussion on the role of WBC and fibrinogen in the pathogenesis of FLHS is further discussed in Chapter 7.

6.5.5 Clinical biochemistry

In order to fully assess liver health, responses of plasma metabolites, CHOL, TG, TP, and AST, were measured (see Figures 6.3, 6.4, 6.5 and 6.6). The data show that E2 induced hypercholesterolemia and hypertriglyceridemia which were associated with severe impairment of liver histology (fat accumulation, inflammation and cell-vacuolisation) (Figures 6.8b, 6.9a,b and 6.10a,b). Plasma AST and TP were also significantly affected in E2 treated birds (especially in restricted feed intake hens), probably reflecting the consequences of an increased deposition of TG and CHOL in the liver of birds temporarily impacting hepatic protein synthesis (Limdi and Hyde, 2003). It should be noted that, when a liver is partially damaged and hepatocytes are filled with fat droplets, swelling and necrosis may occur. However the mitotic activity in the remaining cells begins as early as 12 hours later, eventually restoring the liver to its normal weight (Bertalanffy et al., 1970; Michalopoulos, 2014; Sidorova, 1962). This remarkable capacity to regenerate after injury and the large reserve capacity of the liver tissue make it technically difficult to detect clinical changes and diagnose liver diseases. Many previous investigators have recommend measurements of plasma enzymes to test for FLHS (Diaz et al., 1999; Pearce, 1980; Walzem et al., 1993). However, as these investigators suggest, these tests are indicative of liver damage in birds, and do not specifically demonstrate the presence of liver damage due to FLHS. In this study both GGT and AST were measured, but only AST was significantly reduced in E2 treated hens, reflecting the situation was associated with a disordered metabolic state and liver dysfunction.

This study confirmed the important involvement of oestrogens in lipid metabolism and the disturbance of the lipid metabolism due to elevation of E2 associated with an increase in positive energy balance. Most importantly, the results suggest that the inflammatory response is a contributor to the pathogenesis of FLHS. Further studies are required to explore the interaction of these pathways, especially the role of inflammation.
CHAPTER 7

IN Volvement of the Inflammatory Response in the Pathogenesis of FLHS

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7.1 Introduction

As discussed in the literature review, evidence has accrued in recent years showing that the dysfunction of metabolic processes and immune response pathways underlies many chronic metabolic diseases, including non-alcoholic fatty liver diseases (NAFLD) that occurs in man as a consequence of acquired or inherited overeating in the setting of reduced physical activity (Hotamisligil, 2006; Hotamisligil and Erbay, 2008; Osborn and Olefsky, 2012). It is also well established that obesity in humans is associated with a state of chronic low-grade inflammation
(metabolic inflammation) that is a major player in the link between metabolic syndrome and fatty liver disease (Cusi, 2012; Wisse, 2004). Fatty liver and FLHS are similar metabolic disorders that are associated with comparable factors such as diet, increased feed intake, and lack of exercise.

Inflammation is known to be a defence mechanism triggered when innate immune cells detect infection or tissue injury (Newton and Dixit, 2012). Although inflammation itself is a very complex process and involves many cell types and molecules, a number of the participating molecules are multifunctional and contribute to the inflammatory response at different stages, such as, the initiation, the control during the progression, and its resolution (Serhan, 2011). The main function of inflammation is to resolve the infection or the injurious process, facilitate repair, and return to a relative state of homeostasis. The ideal inflammatory response is rapid and destructive (when necessary), yet specific and self-limiting (Barton, 2008). During the early inflammatory response, immediate pro-inflammatory mediators are released, which initiate and enhance the acute phase response (Gruys et al., 2005). The acute phase response results in a complex systemic reaction associated with the production of markers of inflammation: acute phase proteins (APP) or blood proteins, and immune cytokines (Cray et al., 2009). The APPs are primarily synthesized by hepatocytes and have defensive and repair functions to reduce pathologic damage, promote healing and restore homeostasis (Gruys et al., 2005). In addition, the immune cytokines secreted by a variety of cell types (polymorphonuclear leukocytes, fibroblasts, endothelial cells, monocytes, lymphocytes) play an essential role, not only in regulating inflammatory processes but also in modifying a variety of normal physiologic and metabolic processes (Schett et al., 2013).

There is accumulating evidence indicating that adipose tissue is an endocrine organ capable of synthesizing a number of biologically active compounds that regulate metabolic homeostasis (Coelho et al., 2013). Both the adipocytes and inflammatory cells (that migrate in adipose tissue and/or liver during the acute phase response) secrete numerous pro-inflammatory cytokines. In particular, the intra-abdominal WAT is strategically located to flood the liver with pro-inflammatory cytokines via the portal circulation, and alter hepatic protein synthesis (Libby et al., 2010). In addition, lipid droplets in hepatocytes, like adipocytes, are seen as inducible organelles with roles in cell signalling, regulation of lipid metabolism, membrane trafficking, and control of the synthesis and secretion of inflammatory mediators (Anderson and Borlak, 2008; Arisqueta et al., 2013; Bozza and Viola, 2010). Presumably, hepatocyte lipid droplets could be implicated in impairment of hepatic protein synthesis. Among the proteins of the acute phase response affected by inflammatory cytokines is fibrinogen. Fibrinogen is a clotting factor that provides a substrate for fibrin formation and thus is important in tissue repair. Moreover, fibrinogen plays a multifaceted
role in the immune and inflammatory response (Mosesson et al., 2001). It regulates leukocyte function in inflammation including cell adhesion, migration, cytokine and chemokine expression, degranulation, and other specialized processes (Flick et al., 2004; Ugarova and Yakubenko, 2001).

As mentioned above, the role of the inflammatory response is to resolve infection and injury. Nevertheless, increasing evidence indicates that low-grade chronic inflammation may be the cause of several conditions and diseases associated with obesity, including type-2 diabetes, cardiovascular diseases, and fatty liver disease (Paschos and Tziomalos, 2012). In the previous chapter, it was demonstrated that hens treated with E₂ showed an increased inflammatory response as demonstrated by changes in peripheral blood (elevated leukocyte numbers and fibrinogen levels) and structural alterations of liver tissue. This study suggested that, in addition to the metabolic pathways (altered metabolic profiles), the inflammatory and immune pathways were involved in the hen’s response to elevated oestrogen levels.

A question that arose from Chapter 6 was to what extent does inflammation (as a part of acute phase response) contribute to the progression (pathogenesis) of fatty livers, and most importantly to the precipitation of FLHS outbreaks (liver rupture and haemorrhage as an endpoint of this condition). Perhaps, in the field hens are exposed to an inflammatory insult, in addition to E₂ to develop FLHS. Lipopolysaccharide (LPS) is an endotoxin (the major outer membrane constituent of gram-negative bacteria) and a potent inducer of inflammation (Kaisho and Akira, 2002). Challenging hens with LPS from Escherichia coli would induce low-level hepatic inflammation and mimic the challenges experienced by commercial laying hens.

The aim of this study was to investigate the effect of challenging hens with E₂ and LPS to allow examination of metabolic and inflammatory markers in a controlled manner. The objectives of this study were:

i) To evaluate important markers of inflammation e.g., peripheral leukocyte, plasma fibrinogen levels, and mRNA expression levels of proinflammatory cytokines in hepatocytes, and examine the progression of liver injury (through necropsy and histological examination).

ii) To establish a relationship between liver inflammation (i.e. the expression of mRNA proinflammatory cytokines levels in the hepatocytes) and the incidence of FLHS in hens treated with E₂, LPS and E₂&LPS.
7.2 Materials and Methods

7.2.1 Laying hens and housing conditions

In terms of birds, housing conditions and the diet this experiment was a repetition of the oestradiol hen model (see Chapter 6.2.1 for more details).

7.2.2 Experimental design

The justification for using $E_2$ & LPS

The rationale for using LPS as an inflammatory factor involved in the FLHS-hen model was based on the previous study (Chapter 6), where an increased inflammatory response was demonstrated in experiments with oestradiol. Moreover, studies with human and mouse models had confirmed that circulating endotoxin levels were elevated in patients with NAFLD (Harte et al., 2010). It was suggested that LPS, oxidative stress, cytokine production and other proinflammatory mediators each play roles in delivering a ‘second hit’ during the transition from simple steatosis to NASH (Farrell and Larter, 2006). It is recognised that LPS facilitates a rapid reaction to infection via stimulation of toll-like receptors (TLRs), and activation of the innate immune pathways (i.e. it represents the first line of defence against gram-negative infections) (Kaisho and Akira, 2002). The liver is one of the LPS target organs (Pagani et al., 2003), and it has been also established that it is the clearance organ for LPS (Mimura et al., 1995) and inflammatory mediator production (Pagani et al., 2003).

The hypothesis in the present study, was that hens treated with $E_2$ and LPS will exhibit more rapid onset and greater inflammatory responses, and experience higher incidence of FLHS in terms of liver tissue alterations, than control hens or hens treated with $E_2$ only.

Details of the $E_2$ and LPS hen model

The $E_2$ hen model was induced in 30-week-old Hi-sex laying hens (see Chapter 6). Birds were housed in a temperature-controlled shed with a 16 h light: 8 h dark cycle and had ad libitum access to water and food. Hens were randomly assigned to 6 treatments with 18 replicates for each treatment (n=18). All groups were fed a commercial layer diet during 3 weeks of experimentation (for diet ingredients see Appendix 2).
Birds treated with oestradiol were injected with exogenous $E_2$, 5mg/kg body weight i.m. every 4 days (day 1, 5, 9, and 13) (Table 7.1). For more details on $E_2$ treatment see Chapter 6.2.2. For the LPS treatments, hens were injected intravenously (i.v.) with a single moderate dose of 8 mg/kg body weight of ultra-pure *E. coli* 0111:B4 strain LPS (InvivoGen, San Diego, CA, USA). Oestradiol was administered dissolved in 0.5 mL corn oil/kg BW, while for LPS injections 0.5 mL of PBS /kg BW was used. Birds in the control group were not treated, whereas birds in control-treated groups were injected with 0.5 mL vehicle alone (oil or saline solution, respectively).

**Table 7.1** Summary of the treatments and samples collected for the $E_2$&LPS hen model experiment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment /day</th>
<th>Dose/route</th>
<th>Samples /time</th>
<th>Performance records $^{3,4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Untreated</td>
<td>Nothing</td>
<td>Blood, Liver; 0 h, 3h, 24 h, 1 wk, 2 wk</td>
<td>HDP% Mortality: daily BW: 24h, weekly</td>
</tr>
<tr>
<td>Saline</td>
<td>PBS</td>
<td>0mg/0.5 mL PBS/kg BW, i.v.</td>
<td>The same</td>
<td>The same</td>
</tr>
<tr>
<td>Oil</td>
<td>Oil</td>
<td>0mg/0.5 mL oil/kg BW, i.m.</td>
<td>The same</td>
<td>The same</td>
</tr>
<tr>
<td>$E_2$</td>
<td>$E_2$</td>
<td>5mg/0.5mL oil/kg BW, i.m.</td>
<td>The same</td>
<td>The same</td>
</tr>
<tr>
<td>LPS</td>
<td>LPS</td>
<td>8mg/0.5mL PBS/kg BW, i.v.</td>
<td>The same</td>
<td>The same</td>
</tr>
<tr>
<td>$E_2$ &amp; LPS</td>
<td>$E_2$&amp;LPS</td>
<td></td>
<td>The same</td>
<td>The same</td>
</tr>
<tr>
<td>LPS: Day 1</td>
<td>8 mg/0.5 mL PBS/kg BW, i.v.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_2$:Day 1,5,9,13</td>
<td>5mg/0.5 mL oil/kg BW, i.m.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$E$_2$ = oestrogen-treated; LPS = LPS-treated; $E_2$ & LPS-treated; Oil = corn-oil treated; Saline = PBS-treated;

$^2$Last treatments were on day 13; samples and records were taken on day 14 or 2 wk after first treatment with $E_2$ or LPS.

$^3$Feed was provided *ad libitum* to all groups;

$^4$%HDP = % hen day production; BW = body weight; i.m. = intramuscular; i.v. = intravenous.
7.2.3 Sampling and tests

Blood samples were collected at 3 h, 24 h, and on day 7 and 14 post first treatments with E₂ and LPS injections. At each sampling point, three hens per treatment were euthanised for necropsy records and liver samples. Other hens in the experiment were observed for their general health and welfare status; all hens were euthanised on day 14 of the experiment.

**Blood samples**

Blood samples were taken using individual EDTA vacuntainers and individual blood tubes from the wing vein. Each bird was appropriately restrained to ensure as little stress as possible on the bird. Whole blood was used to measure haematological parameters in an automated analyser (CELL-DYN® System 3700CS, Abbott Park, IL 60064), including the total number of red blood cells (RBC), the total number of white blood cells (WBC) and percentages of white blood cell differential counts.

For other analyses, blood was centrifuged (1,500 rpm for 10 min) and plasma was stored at -20°C. Blood concentrations for plasma metabolites were determined for CHOL, TG, TP, and GGT using commercial kits and a chemistry system (VetTest chemistry analyser, IDEXX Laboratories, Inc. USA). For detailed information on chemical analyses see Chapter 3.3.2. In addition to these metabolites, measurements of plasma fibrinogen levels were carried out. Plasma fibrinogen content was determined by the heat precipitation method (Schalm, 1980) (see Chapter 3.2.2).

**RNA extraction and real-time quantitative RT-PCR**

The mRNA expression levels of IL-1β, IL-6, IL-18 were measured using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays. Three, 30 mg pieces of liver were immediately placed into RNA-Later and stored at -20°C prior to RNA purification. Total RNA was extracted from liver samples using an RNeasy plus mini kit (Qiagen, Doncaster, VIC Australia), following the manufacturer’s directions. Isolated RNA was eluted in 50 ml RNase-free water, and stored at -80°C until use. The yield of total RNA was determined using absorption of light at 260 and 280 nm in a Nanodrop (ND-1000) spectrophotometer.

Primers and probes for the house keeping gene (28S) and target genes (encoding IL-1β, IL-6, and IL-18) were designed, based upon sequences available from public databases, and synthesised by a commercial laboratory (OPERON Technologies, Cologne, Germany). All primers and probes tested
in this study are presented in Table 7.2. All probes were labelled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5’ end and with the quencher N, N, N, N’-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3’ end. Reverse transcription and PCR were performed in separate reactions as previously described (Shini et al., 2010). First, 2 μg of RNA were reverse-transcribed into cDNA in a 20 μl RT reaction using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) in a GeneAmp® PCR system 9700 (Applied Biosystems, Australia). After template denaturation at 65°C for 5 min, 10 μl of cDNA Synthesis Mix were added per tube and the program was continued at 55°C for 50 min, 85°C for 5 min and then held at 4°C until removal from the machine. The PCR was performed in a 10 μl reaction containing 2 μl of cDNA from the RT reaction, 5 μl TaqMan® Universal PCR Master Mix (Applied Biosystems, Australia), 2 μl primer (at 3 μmol) and 1 μl probe (at 1.5 μmol). Each PCR plate contained target genes and 28S rRNA in triplicate and a no-template negative control, containing 2μl water instead of cDNA. The real-time reactions were carried out on an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems, Australia) with the following cycle profile: 95°C for 10 min, and 50 repeats cycles of 95°C for 15 s and then 60°C for 1 min.

Quantification was based on the increased fluorescence detected by the 7900 Fast Sequence Detection System due to hydrolysis of the target-specific probes by the 50 nuclease activity of the rTth DNA polymerase during PCR amplification. Results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye passes a significance threshold (Rn). To account for variation in sampling and RNA preparation, the Ct values for cytokine-specific product for each sample were standardised using the Ct value of 28S rRNA product for the same sample. To normalise RNA levels between samples within an experiment, the mean Ct value for 28S rRNA-specific product was calculated by pooling values from all samples in that experiment. Tube-to-tube variations in 28S rRNA Ct values about the experimental mean were calculated. The slope of the 28S rRNA log10 dilution series regression line was used to calculate differences in input total RNA. Using the slopes of the respective cytokine and chemokine or 28S rRNA log10 dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust cytokine-specific Ct values, as follows:

**Corrected Ct value = Ct \left( N_t - C_{t'} \right) S/S'**

where: 
- \( C_t \) = mean sample Ct;
- \( N_t \) = mean experimental 28S;
- \( C_{t'} \) = mean 28S of sample;
- \( S \) = cytokine slope, and \( S' = 28S \) slope;

Results are expressed as differences (n-fold) between treated samples and controls.
Table 7.2 Primer and probe sequences for qRT-PCR.

<table>
<thead>
<tr>
<th>Target RNA</th>
<th>Probe/Primer</th>
<th>Sequence</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>28S</td>
<td>P</td>
<td>5-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3</td>
<td>X59733</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5-GGCGAAGCCAGAGGAACG-T3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5-GACGACCAGATTTGCAGTC-T3</td>
<td></td>
</tr>
<tr>
<td>IL-1b</td>
<td>P</td>
<td>5-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3</td>
<td>AJ245728</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5-GCTCTACATGTCGTTGATGAGGTAG-3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5-TGTCGATGTCGCCCATGA (FAM)-3</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>P</td>
<td>5-(FAM)-AGGAGAAATGCTGACGAAGCTCTCCA-(TAMRA)-3</td>
<td>AJ250838</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5-GCTCGCCGGCTTCCA-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5-GGTAGGCTCTGAAAGCGAAGAG-3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-GGTAGGCTCTGAAAGCGAAGAG-3</td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>P</td>
<td>5-(FAM)-TCTTTACCAGCGTCCTACCTTCGCACCA-(TAMRA)-3</td>
<td>AJ009800</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5-GCCCTCCTCGGTTTTCAG-3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5-TGGCACCAGCTCATT-3</td>
<td></td>
</tr>
</tbody>
</table>

1P, probe; F, forward primer; R, reverse primer.
**Mortality rate and causes of mortality**

Necropsies were conducted at each sampling point or when hens were found dead or in distress and immediately euthanised.

**Liver histology**

The liver was removed, weighed and individually examined for the presence of haemorrhagic lesions (for details see Chapter 5.3.3). Liver samples were taken for mRNA extraction and histology slides. As described in Chapter 3.3.4, liver samples (small pieces) were fixed in 10% formalin and embedded in paraffin. Sections measuring 5µm were cut and stained with H&E. Liver slides were examined blind by two independent observers.

**Performance parameters**

Egg production and mortality records were taken as per experimental design (see Table 7.1), and %HDP and mortality were calculated as per Chapter 3.2.3 and Chapter 4.2.3, respectively. Body weights were recorded at day 1 time 0 h and then on days 7 and 14.

7.3 **Statistics**

All biochemical analyses and blood cell counting were done in duplicate for each time point. The mean values (±SEM) of plasma metabolites, WBC counts and performance parameters of controls and treated chickens were determined by one-way ANOVA for repeated measures (SAS Institute Inc, 2001). Differences were further separated using Duncan’s multiple range test and considered significant at \( P < 0.05 \). Correlations between different significant measures were determined using Pearson's correlation coefficient.

For cytokine mRNA expression, statistical analyses were performed using Microsoft Office Excel 2010 v14.0 (Microsoft Corporation). PCR tests were repeated two times and each sample was assayed in triplicate. Data from two repeated experiments were pooled for presentation and statistical analysis. The mean and standard error of the mean (mean ± SEM) for all cytokines were calculated for each group (controls and treatments). For statistical purposes, an unpaired \( t \)-test was used to compare two means (control vs. E\(_2\) treatment; control vs. LPS treatment and E\(_2\) vs. LPS treatment) and determine the \( P \) value. A 99% confidence interval for the true difference between the means was set, and the values were considered significant at \( P < 0.01 \).
7.4 Results

As demonstrated in Chapter 6, the E2 model induced FLHS in laying hens and this was exacerbated by a challenge with LPS (see section 7.4.5 below).

7.4.1 Cytokine gene expression in the liver

Data on mRNA expression of targeted cytokines (IL-1β, IL-6, and IL-18) from hepatocytes are presented at 3h and 24 h after first treatment with E2, LPS and E2 & LPS (Figure 7.1). These two sampling points are relevant in terms of acute changes post LPS and E2 administrations, and were expected to reflect changes in mRNA expressions of selected inflammatory cytokines. Figure 7.1 shows the standardised data for cytokine levels expressed as fold changes in mRNA levels compared to basal mRNA levels. The mean mRNA levels of IL-1β and IL-6 of each group/treatment pooled together increased by 3 to 58 folds compared with basal levels (P<0.01) at 3 h following treatments with E2 and LPS. Both, IL-1β and IL-6 mRNA expression were significantly (P<0.01) elevated in E2, LPS and E2 & LPS hens (Appendix 5, Table 6).

The expression of IL-6 mRNA in hepatocytes in all treated groups was elevated (P<0.01) from 6 to 56 folds as compared to baseline and controls. The highest fold change was found in LPS and E2 & LPS treated birds at 3 h post-treatments when compared to controls. At 24 h later IL-6 gene expression was slightly decreased, however was still significantly elevated approximately by 20 folds. When compared to E2 treatments all LPS and E2 & LPS birds demonstrated higher (P<0.01) levels of IL-6 and IL-1β. The expression of mRNA for IL-6 and IL-1β in E2 treated birds was moderate but still significantly (P<0.01) up-regulated from 3 to 12 folds at 3h and 24 h after treatments with E2, respectively.

The mRNA levels for IL-1β were better expressed at 24 h in all treated birds (E2, LPS and E2 & LPS). At 3 h and 24 h, mRNA levels for IL-1β were highly (P<0.01) up-regulated in E2 & LPS treated birds when compared to other treatments (E2 and LPS). The expression of IL-18 mRNA in the liver tissue was lower than IL-1β and IL-6 mRNAs in all treated birds. At 3 h and 24 h post treatment with E2 the mRNA levels for IL-18 were found minimal to undetectable in E2 treated birds. In LPS-treated birds at 3h and 24h the expression of mRNA IL-18 was significantly (P<0.01) elevated (<5 folds).
Figure 7.1  Cytokine mRNA expression profiles from real-time qRT-PCR analyses of hepatocytes of treated birds compared to baseline levels and controls at 3 h and 24 h post-treatments.

E2 = oestrogen-treated; LPS = LPS-treated; E2&LPS = E2- and LPS-treated;
All Ct values were corrected using the housekeeping gene 28S, and time point 0 was used as the calibrator. Values are expressed as mean ± SEM fold change relative to control (data is pooled for all controls: not treated, corn-oil treated and PBS-treated birds). Error bars show SEM from triplicate samples (n=16) from two separate qRT-PCR experiments (P<0.01, unpaired t-test).

7.4.2 Blood cell counts

Data on blood parameters (WBC and RBC counts, and percentages of heterophils and lymphocytes and H/L ratios) are presented in Table 7.3.

At 3 h post-treatments (with E2, LPS and E2 & LPS), WBC counts were significantly increased (P<0.05) in E2 and LPS treated birds when compared to controls but not E2 & LPS treated birds. As shown in Table 7.3, from the differential counts (%) of heterophils and lymphocytes and H/L ratios, the increase in leukocytes included both, heterophils and lymphocytes. Other WBC types did not
show any significant change in percentages (data not shown), while thrombocytes percentages were measured inconsistently therefore were not included in the study.

At 24 h post-treatments, hens exposed to LPS had a numerical increase in WBC but at this time the increase was in favour of heterophils. There was a significant \((P<0.05)\) increase in heterophil percentages and H/L when compared to controls and measurements at 3 h (1.42 vs. 0.48 or 0.43, respectively). Similarly, leukocyte numbers were significantly raised \((P<0.01)\) in birds treated with \(E_2\) & LPS, while birds treated with only \(E_2\) continued to have elevated WBC numbers.

Birds treated with \(E_2\) or \(E_2\) & LPS demonstrated high numbers of leukocytes at week 1 and 2 post-treatments. At week 1 this increase in leukocytes included mainly heterophil numbers, but at week 2 the increase was proportional therefore the H/L ratio was decreased and was found to be slightly significantly different \((P<0.05)\) from LPS treated birds but similar to values at 3 h (Table 7.3). One week after treatments started, WBC of birds treated with LPS were still elevated (approx. \(32\times10^9/L\)), but decreased significantly \((P<0.01)\) and reached the level of the control birds (approx. \(18.6\times10^9/L\)) at week 2.

Data on RBC counts on treated and untreated birds are presented in Table 7.3. As shown in the results, the average of RBC counts was more consistent in all birds (untreated and treated). There was a slight but significant increase \((P<0.05)\) in RBC counts in control birds at 24 h and in \(E_2\) treated birds at week 2 compared to LPS treated birds at 3 h and at week 1.
Table 7.3  Changes in blood cell counts (WBC and RBC), heterophil and lymphocyte percentages and H/L ratios in control and treated laying hens\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time</th>
<th>WBC\textsuperscript{2} (x10\textsuperscript{9}/L)</th>
<th>Heterophils (%)</th>
<th>Lymphocytes (%)</th>
<th>H/L ratio</th>
<th>RBC\textsuperscript{2} (x10\textsuperscript{12}/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>3 h</td>
<td>18.0\textsuperscript{a}</td>
<td>29.6\textsuperscript{a}</td>
<td>61.9\textsuperscript{a}</td>
<td>0.48\textsuperscript{a}</td>
<td>2.5\textsuperscript{a,b}</td>
</tr>
<tr>
<td>E\textsubscript{2}</td>
<td>3 h</td>
<td>25.7\textsuperscript{b}</td>
<td>28.0\textsuperscript{a}</td>
<td>62.2\textsuperscript{a}</td>
<td>0.45\textsuperscript{a}</td>
<td>2.3\textsuperscript{a}</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>27.0\textsuperscript{b}</td>
<td>28.2\textsuperscript{a}</td>
<td>65.4\textsuperscript{a}</td>
<td>0.43\textsuperscript{a}</td>
<td>2.1\textsuperscript{a}</td>
</tr>
<tr>
<td>E\textsubscript{2} &amp; LPS</td>
<td>21.7\textsuperscript{a,b}</td>
<td>26.7\textsuperscript{a}</td>
<td>64.3\textsuperscript{a}</td>
<td>0.42\textsuperscript{a}</td>
<td>2.2\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>24 h</td>
<td>17.5\textsuperscript{a}</td>
<td>25.6\textsuperscript{a}</td>
<td>63.1\textsuperscript{a}</td>
<td>0.41\textsuperscript{a}</td>
<td>2.6\textsuperscript{b}</td>
</tr>
<tr>
<td>E\textsubscript{2}</td>
<td>24 h</td>
<td>28.2\textsuperscript{b}</td>
<td>29.0\textsuperscript{a}</td>
<td>64.1\textsuperscript{a}</td>
<td>0.45\textsuperscript{a}</td>
<td>2.2\textsuperscript{a}</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>35.8\textsuperscript{b,c}</td>
<td>53.6\textsuperscript{c}</td>
<td>37.8\textsuperscript{b}</td>
<td>1.42\textsuperscript{c}</td>
<td>2.3\textsuperscript{a}</td>
</tr>
<tr>
<td>E\textsubscript{2} &amp; LPS</td>
<td>43.8\textsuperscript{b,c}</td>
<td>55.6\textsuperscript{c}</td>
<td>36.2\textsuperscript{b}</td>
<td>1.54\textsuperscript{c}</td>
<td>2.2\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>1 wk</td>
<td>18.7\textsuperscript{a}</td>
<td>22.6\textsuperscript{a}</td>
<td>66.1\textsuperscript{a}</td>
<td>0.34\textsuperscript{a}</td>
<td>2.4\textsuperscript{a,b}</td>
</tr>
<tr>
<td>E\textsubscript{2}</td>
<td>1 wk</td>
<td>42.7\textsuperscript{b,c}</td>
<td>35.9\textsuperscript{b}</td>
<td>54.4\textsuperscript{a,b}</td>
<td>0.66\textsuperscript{b}</td>
<td>2.3\textsuperscript{a}</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>31.9\textsuperscript{b}</td>
<td>48.8\textsuperscript{h,c}</td>
<td>38.0\textsuperscript{b}</td>
<td>1.28\textsuperscript{c}</td>
<td>2.0\textsuperscript{a}</td>
</tr>
<tr>
<td>E\textsubscript{2} &amp; LPS</td>
<td>34.8\textsuperscript{b,c}</td>
<td>51.6\textsuperscript{c}</td>
<td>42.9\textsuperscript{b}</td>
<td>1.20\textsuperscript{c}</td>
<td>2.5\textsuperscript{a,b}</td>
<td></td>
</tr>
<tr>
<td>CONT</td>
<td>2 wk</td>
<td>15.9\textsuperscript{a}</td>
<td>29.4\textsuperscript{a}</td>
<td>59.4\textsuperscript{a}</td>
<td>0.49\textsuperscript{a}</td>
<td>2.2\textsuperscript{a}</td>
</tr>
<tr>
<td>E\textsubscript{2}</td>
<td>2 wk</td>
<td>52.3\textsuperscript{c}</td>
<td>36.1\textsuperscript{b}</td>
<td>55.5\textsuperscript{a,b}</td>
<td>0.65\textsuperscript{b}</td>
<td>2.6\textsuperscript{b}</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>18.6\textsuperscript{a}</td>
<td>22.0\textsuperscript{a}</td>
<td>66.5\textsuperscript{a}</td>
<td>0.33\textsuperscript{a}</td>
<td>2.4\textsuperscript{a,b}</td>
</tr>
<tr>
<td>E\textsubscript{2} &amp; LPS</td>
<td>35.5\textsuperscript{b,c}</td>
<td>30.3\textsuperscript{b}</td>
<td>58.2\textsuperscript{a}</td>
<td>0.52\textsuperscript{b}</td>
<td>2.2\textsuperscript{a}</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1}E\textsubscript{2} = oestrogen-treated; LPS = LPS-treated; E\textsubscript{2}&LPS = E\textsubscript{2}- and LPS-treated; CONT = data is pooled for all controls (not treated, corn-oil treated and PBS-treated birds); WBC=White blood cells; RBC=Red blood cells;

\textsuperscript{2}Values are expressed as mean (n=6). Means with different superscripts (a-c) within a column are significantly different (P <0.05).
7.4.3 Fibrinogen

As shown in Figure 7.2, at 3 h plasma fibrinogen levels in LPS and E₂ & LPS treated birds were slightly elevated ($P<0.05$) when compared to control birds (3.8 and 3.6 g/L vs. 2.0 g/L, respectively). At 24 h there was a high response in fibrinogen levels in all treated birds (E₂, LPS and E₂ & LPS), surprisingly in E₂ treated hens the value of fibrinogen concentration was higher than in LPS treated hens (5 g/L vs. 4.1 g/L, respectively). Fibrinogen concentrations rose earlier in LPS treated birds compared to E₂ treated birds. However, as shown in Figure 7.2, elevated concentrations of fibrinogen remained high in weeks 1 and 2 post-treatments with E₂, while in LPS treated birds, fibrinogen levels were reduced to control values after the first week of treatments. As in the first experiment with E₂ (Chapter 6), fibrinogen levels correlated with WBC levels in E₂ treated birds. Thereafter, plasma fibrinogen concentrations declined and were found lower in LPS treated birds than the values of control birds, however this difference was statistically not significant.

**Figure 7.2** Plasma fibrinogen concentrations (g/L) in treated and untreated birds.

$E₂ = $ oestrogen-treated; $LPS = $ LPS-treated; $E₂&LPS = $ E₂- and LPS-treated; control $=$ data is pooled for all controls (not treated, corn-oil treated and PBS-treated birds); Values are expressed as mean ±SEM (n=6).
7.4.4 Plasma metabolites and gamma glutamyl transferase levels

Plasma concentration for TG and GGT for treated (E₂, LPS, and E₂ & LPS) and untreated birds, are presented in Figure 7.3 and 7.4, respectively. Other metabolites (AST, CHOL, TP, and GLU) are presented in Appendix 8, Table 7. Data from control groups are pooled and presented together for control (untreated) and oil and saline treated groups.

As demonstrated in the E₂ hen model experiment, hens treated with E₂ showed again increased ($P<0.01$) levels of plasma lipids especially TG (Figure 7.3); CHOL was less elevated than TG in E₂ treated birds (Appendix 8, table 7). In both E₂ treated groups plasma TG rose significantly 24 h after first treatment, and then continued to be increased at week 1 post-treatments. Birds treated with LPS indicated a significant decrease ($P<0.05$) in plasma lipids, especially at 24 h post treatments when compared to control birds. For this study, both GGT and AST levels were measured, as both enzymes could indicate liver damage. Data are presented here for GGT concentrations (Figure 7.4), which demonstrate a slight significant increase ($P<0.05$) of the levels in particular within 3 h after first treatments with E₂ and LPS. Plasma concentrations for TP, and GLU did not show any significant changes in all birds over time (Appendix 8, Table 7). All control birds had insignificant changes in plasma metabolites at all measurement points.
Figure 7.3  Plasma concentrations of TG (IU/L) in treated and untreated birds.

$E_2$ = oestroge-treated; LPS = LPS-treated; $E_2$&LPS = $E_2$- and LPS-treated; control = data is pooled for all controls (not treated, corn-oil treated and PBS-treated birds); TG = Triglycerides; Values are expressed as mean ±SEM (n=6).

Figure 7.4  Plasma concentrations of GGT (IU/L) in treated and untreated birds.

$E_2$ = oestrogen-treated; LPS = LPS-treated; $E_2$&LPS = $E_2$- and LPS-treated; control = data is pooled for all controls (not treated, corn-oil treated and PBS-treated birds); GGT=gamma glutamyl transferase; Values are expressed as mean ±SEM (n=6).
7.4.5 Mortality and necropsy results

Data on mortality and liver haemorrhagic score demonstrated that all birds (100%) treated with E₂ and E₂&LPS developed FLHS (Table 7.4). Birds treated with E₂&LPS showed and more severe signs of FLHS (after first week of treatments all necropsied birds had liver haemorrhagic score = 4-5). However, the mortality rate was higher (P<0.05) in E₂ birds than in E₂&LPS treated birds. There were no mortalities in control-untreated and LPS-treated birds. All control groups had a low incidence of FLHS, in terms of haemorrhagic score (one bird had liver haemorrhagic score 1-2).

Table 7.4 Effect of oestrogen injections, and a single LPS injection on mortality, the incidence of FLHS, and liver haemorrhagic of laying hens.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Number of birds</th>
<th>FLHS (%)</th>
<th>Haemorrhagic score (%)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>54</td>
<td>5.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1-2</td>
<td>0</td>
</tr>
<tr>
<td>E₂</td>
<td>18</td>
<td>66.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4-5</td>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2-3</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>18</td>
<td>5.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2-3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>E₂&amp;LPS</td>
<td>18</td>
<td>88.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4-5</td>
<td>5.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2-3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Data are reported for the whole experimental period; N of birds sacrificed at each sampling point was 3 birds/treatment. At the end of the experimental period, all birds were sacrificed and underwent post-mortem examination.

<sup>2</sup>Controls = untreated; E₂ = oestrogen-treated; LPS = LPS-treated; E₂ & LPS-treated;

<sup>3</sup>There were no significant differences between untreated and oil-treated and saline-treated groups; therefore, data are pooled and presented together;

<sup>4</sup>Data on the occurrence of FLHS are divided to reflect haemorrhagic scores (next column) and so indicate the range and severity of the lesions

<sup>a,b,c,d</sup>Values within a column with different superscripts differ significantly (P<0.05).
7.4.6 Liver histological examination

The liver tissue from hens treated with E2, LPS, and E2 and LPS had significant histological changes when compared to samples from control hens (Figures 7.5, 7.6, 7.7), including the most extensive fat deposition (in terms of cells with 50 to 75% vacuolisation) and WBC and RBC infiltration in the areas surrounding blood vessels or into liver parenchyma. Large vacuoles containing fat distended many hepatocytes (Figure 7.6a,b) or totally replaced their cytoplasm and atrophied their nucleus (Figure 7.7a,b). The pathological evaluation indicated other abnormalities associated with lipid droplets found in the sinusoidal endothelial cells (Figure 7.7a), Disse space (which is located between the sinusoidal membrane and plasma membrane of the liver cell) (Figure 7.6a), perisinusoidal stellate cells or in the spaces of arterioles, venules (see also Figure 6.14). In particular, histological slides from both E2, and E2 & LPS treated hens revealed major areas of mononuclear aggregations, and increased incidence of sinusoidal congestions and petechial haemorrhages. These results corroborate with previous observations from the E2 hen model.
Figure 7.5  Histological sections showing livers from control hens (a) and LPS treated hens (b). Note normal portal triad and hepatocyte fat infiltration (a), (yellow arrow); and inflammatory cell infiltration (green arrow) in the periportal area surrounding portal vein & artery and the bile duct (b); H&E stain, using x400 magnification.
Figure 7.6  Histological sections showing periportal inflammation in livers from E2 & LPS treated hens.
Note moderate periportal inflammation (green arrow) and fat infiltration (yellow arrow); H&E stain, using x400 magnification.
Figure 7.7  
Histological sections showing inflammation and hemorrhages in livers from LPS & E₂ treated hens.  
Note severe parenchymal and sinusoidal fat (yellow arrow), leukocyte (green arrow), RBC (red arrow) infiltration and sinusoidal dilation, congestion and telangiectasias (a & b);  
H&E stain, using x400 magnification.
7.4.7 Performance parameters

Egg weight, body weight and relative organ (liver and spleen) weights did not significantly change in response to treatments (Table 7.5) but there was a slight increase (not statistically significant \(P>0.05\)) in relative liver weight of E2 treated hens at wk 1 and 2, and in relative spleen weight in LPS treated hens when compared to control hens.

**Table 7.5** Egg weight, body weight (g), and relative liver weight (g/100g BW) and relative spleen weight (g/100g BW) in treated and untreated birds.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Time of measurements</th>
<th>Egg weight (g)</th>
<th>SEM</th>
<th>BW (g)</th>
<th>SEM</th>
<th>Relative LW (g/100g BW)</th>
<th>Relative SW (g/100g BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 h</td>
<td>59.1</td>
<td>0.2</td>
<td>1793</td>
<td>40</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td>59.0</td>
<td>0.2</td>
<td>1802</td>
<td>29</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>59.0</td>
<td>2.2</td>
<td>1775</td>
<td>34</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>E2 &amp; LPS</td>
<td></td>
<td>59.3</td>
<td>0.1</td>
<td>1765</td>
<td>31</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Control</td>
<td>24 h</td>
<td>NA</td>
<td>NA</td>
<td>1794</td>
<td>42</td>
<td>2.72</td>
<td>0.10</td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>1803</td>
<td>28</td>
<td>2.75</td>
<td>0.11</td>
</tr>
<tr>
<td>LPS</td>
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<td>NA</td>
<td>NA</td>
<td>1768</td>
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<td>2.80</td>
<td>0.12</td>
</tr>
<tr>
<td>E2 &amp; LPS</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>1748</td>
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<td>3.01</td>
<td>0.12</td>
</tr>
<tr>
<td>Control</td>
<td>1 wk</td>
<td>61.3</td>
<td>0.6</td>
<td>1805</td>
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<tr>
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<td>61.1</td>
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<td>1803</td>
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<td>58.6</td>
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<td>1768</td>
<td>38</td>
<td>3.02</td>
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</tr>
<tr>
<td>E2 &amp; LPS</td>
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<td>58.7</td>
<td>0.6</td>
<td>1739</td>
<td>27</td>
<td>3.62</td>
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<tr>
<td>Control</td>
<td>2 wk</td>
<td>61.3</td>
<td>0.7</td>
<td>1816</td>
<td>34</td>
<td>2.52</td>
<td>0.10</td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td>61.2</td>
<td>1.0</td>
<td>1801</td>
<td>31</td>
<td>3.24</td>
<td>0.13</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>59.5</td>
<td>1.7</td>
<td>1813</td>
<td>27</td>
<td>2.99</td>
<td>0.12</td>
</tr>
<tr>
<td>E2 &amp; LPS</td>
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<td>61.7</td>
<td>1.0</td>
<td>1751</td>
<td>28</td>
<td>3.44</td>
<td>0.13</td>
</tr>
</tbody>
</table>

**BW** = body weight; **LW** = Liver weight; **SW** = spleen weight; **NA** = data not available; 
E2 = oestrogen-treated; LPS = LPS-treated; E2&LPS = E2- and LPS-treated; control = data is pooled for all controls (not treated, corn-oil treated and PBS-treated birds); Values are expressed as mean of all birds in experiment for egg weights and body weights and \(n=3\) for relative LW and SW.
7.6 Discussion

In this study, an E₂ & LPS hen model was employed to investigate the involvement of inflammatory response in the pathogenesis of FLHS. The evaluation of the physiological and metabolic responses was undertaken along with the inflammatory markers of plasma and liver to study the disease as it develops in treated hens. The hepatic mRNA levels of the genes that encode the key regulators of the hepatic inflammation (IL-1β, IL-6 and IL-18) (Braunersreuther et al., 2012) were evaluated to explore their expression in hepatocytes during the inflammatory responses in hens treated with E₂, LPS or both E₂ & LPS. The goal of this study was to understand the involvement of inflammatory response in the pathogenesis of FLHS in laying hens; therefore the discussion here will focus on the inflammatory parameters evaluated in this study.

There is a large number of cytokines that are involved in the regulation of endocrine-metabolic and immune responses that are produced by leukocytes, hepatocytes and adipose tissue. Cytokines targeted in this study (IL-1β, IL-6, and IL-18) are of interest because they are involved in the generation of systemic and local responses to infection and injury by generating fever, activating lymphocytes and by promoting leukocyte infiltration at sites of infection or injury (Finotto et al., 2004). Interleukin -6 (IL-6) stimulates the acute-phase reaction, which enhances the innate immune system and protects against tissue damage (El-Kadre and Tinoco, 2013; Gruys et al., 2005).

Both IL-1β and IL-18 are related cytokines that were recognized early on for their ability to cause a wide variety of biological effects associated with infection, inflammation, and autoimmunity (Dinarello, 2006; Finotto et al., 2004). Both IL-1β and IL-18 are members of the same structural family; IL-1β regulates systemic and local responses to infection, injury and immunological challenge by generating fever, activating lymphocytes and promoting leukocyte transmigration into sites of injury or infection (Dinarello, 2006), while IL-18 is essential to host defences against severe infections, in particular, the clearance of intracellular bacteria, fungi and protozoa (Nakanishi et al., 2001). IL-18 should be seen as a unique cytokine that enhances innate immunity and both Th1- and Th2-driven immune responses.

In this study, the expression of IL-1β, IL-6, and IL-18 mRNA in hepatocytes of laying hens were quantified using real-time qRT-PCR. As presented in the results (Figure 7.1), the expression of both IL-1β, IL-6 were highly upregulated in both E₂ and LPS treated hens, with IL-6 responding stronger in the acute phase response (3 h post-treatments) and IL-1β being more expressed at a later stage (at 24 h). It is recognised that cytokines crosstalk on multiple levels. Cellular signalling
studies have shown that IL-1β counteracts signal transduction of IL-6-type cytokines at different levels: it affects IL-6-induced gene expression by acting on target gene promoters (Radtke et al., 2010). This mechanism explains observations in the current study that indicated a down-regulation of the IL-6 mRNA expression while levels of IL-1β mRNA were highly up-regulated. Moreover, it has been suggested that IL-6 has anti-inflammatory properties. IL-6 appears to be the primary inducer of the hepatocyte-derived acute-phase proteins, many of which have anti-inflammatory properties (Barton, 1997).

In terms of the treatments, LPS is known to lead to an increased inflammatory response and up-regulation of IL-6, IL-1β mRNA expression in hepatocytes. The administration of E2 only moderately stimulated the expression of IL-6, IL-1β, but the combination of E2 with LPS caused a higher and stronger response than (E2 alone) and longer than LPS alone, thus reinforcing the hypothesis that the inflammatory response (induced by a bacterial toxin) is involved in triggering liver injury in the context of hepatic steatosis.

Lipopolysaccharide is a molecule that induces local and systemic inflammation and is associated with progression of liver injury (Harte et al., 2010). Bacterial LPS (endotoxin) has been implicated in the pathogenesis of acute liver disease through its induction of the pro-inflammatory cytokines. Evidence suggests that endotoxin-induced cytokines are important mediators of hepatic steatohepatitis. In relation to NAFLD, it has been shown that saturated fatty acids represent an endogenous danger in the form of a first hit, thereby inducing sensitization to LPS-induced inflammasome activation and inflammatory injury (Bieghs and Trautwein, 2013).

Many studies have supported a possible role for endotoxin in metabolic diseases. In humans, it has been confirmed that intestinal permeability and small intestinal bacterial overgrowth are increased in NAFLD patients and that these factors are associated with the severity of hepatic steatosis (Miele et al., 2009). In FLHS in laying hens, this is a new area that could be explored to determine how the gut microflora could influence the progression of hepatic lipidosis and an outbreak of FLHS in individuals with increased metabolism (BW and egg production). Recent studies show that intestinal flora function much like a metabolic “organ,” influencing nutrient acquisition, energy homeostasis, and, ultimately, the control of body weight (Frazier et al., 2011). An increased gut permeability, low-grade endotoxemia, and fatty liver are observed in animal models of obesity caused by either high-fat or high-fructose feeding. The role of the gut microbiota and metabolic endotoxemia-induced inflammation in the development of FLHS needs to be explored further. It seems that feed (or nutrition) including lipids and other molecules of lipid precursors, microflora,
and the physiology of intestinal membranes and special structural features of the liver are major players of the inflammatory response starting in the gut and affecting the first and most visited organ, the liver.

Other markers of liver inflammation that were evaluated in this study were peripheral and local (liver tissue) leukocyte response through WBC counts and liver histology. Data on leucocyte counts and percentages supported the hypothesis that a general inflammatory and immune response caused an increase in total leukocyte counts and triggered their movement towards liver tissue, most probably due to release of hepatic cytokines (in the case of E2 injections) or LPS circulation in the liver. One inflammatory molecule that has not previously mentioned in the pathology of FLHS is fibrinogen.

Fibrinogen can be used as a general clinical biomarker of inflammation, including cases of liver inflammation and damage. Measurement of the fibrinogen concentration is not a specific test, as it may identify the inflammation or infection but would not identify the source of inflammation within the body. However, elevated levels of fibrinogen 2 to 10 times higher than normal values could indicate the development of cellular and molecular processes involved in the coagulation, pathogenesis of inflammation-related diseases or infectious diseases (Levi et al., 2003). Fibrinogen is an inflammatory molecule that was altered in the experiments with E2 (Chapter 6 and 7) and E2 & LPS (Chapter 7). Elevated plasma levels of fibrinogen, indicating chronic subclinical inflammation, have been associated with metabolic diseases such as NAFLD (Bhatia et al., 2012). The low-grade chronic inflammatory state results in several deleterious pathophysiological processes including abnormal glucose, fatty acid and lipoprotein metabolism, increased oxidative stress, deranged cytokine profile, hypercoaguability, endothelial dysfunction, and accelerated progression of atherosclerosis (Bhatia et al., 2012).

Fibrinogen is considered to be a moderate APP (Cray et al., 2009). It is a slow reacting positive acute phase reactant with a possible delay of some days after infection (Gruys et al., 2005). Compared to other acute phase proteins, fibrinogen levels in humans can be lower but it can persist for a longer time than C-reactive protein and serum amyloid A (see Figure 7.8) (Gabay and Kushner, 1999).
Figure 7.8  Acute phase proteins and the systemic inflammatory response

This makes fibrinogen testing a valuable marker that could help in the detection of low-grade (subacute) inflammation which is now seen as a critical component of the metabolic syndrome (Wisse, 2004). The stimulation of fibrinogen synthesis during acute-phase reaction is mediated by cytokines, and in particular IL-6 has a prominent role in the enhancement of fibrinogen synthesis by directly up-regulating fibrinogen gene expression (Caso et al., 2009). In the current study the evaluation of mRNA expression of IL-6 demonstrated an earlier up-regulation of IL-6 (at 3 h post-treatments with LPS and E2 & LPS) than the fibrinogen levels. Increased levels of fibrinogen followed the IL-6 up-regulation and peaked at 24 h post-treatments. These data demonstrate the importance of acute phase response and involvement of IL-6 and fibrinogen as a protective molecules that participate in the resolution of inflammation and tissue repair (Liaskou et al., 2012).

What causes hepatic inflammation, and how can inflammation be controlled and prevented, so that fatty livers do not have haemorrhage events? These are some questions that have yet to be answered. It is very important, therefore, to develop new biomarkers for the detection of birds predisposed to FLHS.
CHAPTER 8

GENERAL DISCUSSION AND IMPLICATIONS OF THE RESEARCH

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8.1 Introduction

Advances in genetic selection, nutrition, and other management practices have contributed to the creation of the “modern laying hen” with improved egg production characteristics, and a liver which must accommodate increased metabolic demands. The liver is the major site of synthesis of lipids and the majority of proteins, including enzymes, hormones, blood proteins, clotting factors, inflammatory and immune molecules are also synthetised in the hepatocytes (see Chapter 2). When hepatic lipogenesis exceeds the capacity of lipid secretion and metabolism, triglycerides accumulate in the liver, causing a fatty liver. High producing laying hens have a fatty liver because of the demands of yolk synthesis. In some situations, this phenomenon can develop into liver damage, liver capsule rupture, haemorrhage and death of the bird, or FLHS. It appears that the balance between hepatic synthesis and secretion of lipids is therefore a key point in the regulation of hepatic and extrahepatic fat deposition in the laying hen. As described in Chapter 2, despite its obvious importance, the aetiology of FLHS has not been well-defined and the pathogenesis is poorly understood.

In this thesis, investigations under field and experimental conditions were undertaken in an attempt to better define the occurrence, aetiology and pathogenesis of this metabolic disorder and open new possibilities for its control and prevention in commercial laying birds.
8.2 Is FLHS still a problem for birds in cages?

The results of the first study, conducted in the layer facility at the Gatton Campus, University of Queensland, showed that housing system did not have any significant effect on hen egg production and mortality rates; however it influenced the BW, and especially affected causes of mortality in hens. The most common cause of death in hens kept in cages was FLHS with 74% of hens dying from this condition. The majority of hens kept in free range died from cannibalism (77%) and cachexia (14%), while hens in barns had problems such as cannibalism (59%), reproductive tract infections (10%) and cachexia. This study is in accord with previous studies that concluded that FLHS is a disease that mainly affects hens kept in cages (Butler, 1976; Couch, 1956; Hansen and Walzem, 1993; Julian, 2005; Leeson, 2007; Neill et al., 1975; Peckham, 1984; Ringer and Sheppard, 1963; Simonsen, 1978; Weitzenburger et al., 2005). Data presented in this thesis also demonstrated that hens in cages had significantly increased body weights. This presumably reflected a positive energy balance induced from a lack of exercise due to restricted space in cages. It appears that monitoring BW throughout the laying cycle may be a helpful diagnostic tool to predict FLHS in a laying flock.

8.2.1 What about Queensland?

The initial “field” investigation in a research facility demonstrated the possible significance of FLHS in caged layers and prompted a more intensive investigation of FLHS in commercial flocks. An epidemiological study was undertaken and involved a questionnaire followed by monitoring of selected laying flocks in South Eastern Queensland. The results suggested that the majority of egg producers were not aware of FLHS despite about 40% of birds dying from FLHS. These results indicate that FLHS remains a significant cause of death of laying hens in Queensland and presumably Australia. It may be that due to the limited number of necropsies carried out and undetected subacute cases, the actual prevalence of FLHS could be higher than reported in this study. Nevertheless, this investigation indicates that FLHS continues to be a disease with a high prevalence in cages, most probably due to housing conditions associated with restricted movements of hens in cages, which predisposes to accumulation of fat in the liver and hepatic rupture.
8.2.2 Temperature and FLHS

The results of the second study revealed that laying hens, in multi-tier cages in a controlled environment (thermo-neutral) shed are at the same risk of developing FLHS as hens kept in naturally ventilated sheds. This is in contrast to most previous studies that found more FLHS deaths at temperature extremes during hot weather (Couch, 1956; Greuel and Hartfiel, 1968; Ivy and Nesheim, 1973; Pearson and Butler, 1978a; Schexnailder and Griffith, 1973). Whether the increased incidence of FLHS is a response to temperature per se, or the response to a stressor, has not been examined. Given the current results and those of Lee et al. (1975) who observed that keeping temperature controlled in the thermo-neutral zone does not decrease the incidence of FLHS suggests that stress rather than ambient temperature is the important factor. Nevertheless, ambient temperature is related to the energy balance, i.e. birds kept in battery cages have lower energy requirements, and if maintained at thermo-neutral temperatures require even less energy, as they do not need the extra energy to activate mechanisms for maintaining body temperature (Silva, 2006). The exposure of birds to cold or heat seems to induce stress and influence lipid metabolism in the chicken.

8.2.3 Other predisposing factors for FLHS

Other factors could have been involved in the aetiology of FLHS. Most investigators agree that lack of exercise is a contributor to FLHS. Commercial laying hens are under the influence of multiple factors that affect their physiology such as restricted movement (associated with increased BW), increased egg production (and levels of oestradiol) and other nutritional and stress factors (including temperature changes) which predispose hens to FLHS. The epidemiological study conducted in this thesis involved measurements of blood parameters so that the hen’s clinical biochemical responses could be explored. Circulating levels of metabolites (CHOL, TG, and GLU and TP) are biomarkers of energy and metabolic responses (Bedogni et al., 2006; Brown et al., 2010).

Circulating levels of TG were significantly increased in hens surveyed in this study, and were associated with increased egg production. It is recognised that laying hens normally have increased circulating lipid metabolites in particular TG from 2-10 fold during egg production (Klasing, 1998), and develop hepatic steatosis to meet the requirements for yolk lipid synthesis (Hansen and Walzem, 1993). Data from the current study revealed that higher levels of TG in hens from 35 to 60 wk of age. In some cases, this increase was associated with a slight rise in GLU concentrations. It
appeared that the diet and level of egg production influenced plasma metabolite levels. It was concluded that results of this study demonstrated that the acute and chronic forms of FLHS are a significant source of losses in egg production for cage egg producers. Factors such as housing and nutrition contribute to increased BW and liver fat deposition, resulting in increased flock deaths from FLHS.

8.3 An E₂ laying hen model to study the aetiopathogenesis of FLHS

Previous studies (see Chapter 2) have implicated oestrogen as a factor in the production of FLHS along with the necessity for the hen to be in a positive energy balance, to facilitate hepatic fat accumulation and the development of FLHS. This suggests that manipulation of these factors may allow the experimental induction of the condition and the development of a reproducible model to study this disease. Moreover, a model would allow the determination of molecular and cellular factors that influence fat accumulation and inflammatory processes in the avian liver. A similar approach has been adopted to allow the examination of obesity and fatty liver pathogenesis in humans (see Chapter 2). These studies suggest that liver is the first organ to be affected when adipose tissue becomes dysfunctional and inflamed, initially undergoing a pro-inflammatory state followed by inflammatory processes.

In Chapter 6 it was demonstrated that the FLHS model developed by Stake et al., (1981) was applicable to the modern layer; this allowed an investigation of the effect of the amount of feed consumed on the occurrence of FLHS. The administration of exogenous E₂ caused the disease in adult (30 wk old) laying hens and was associated with significant changes in liver relative weights, and macroscopic (fat depositions, haemorrhages and haematomas) and microscopic (liver structure) alterations.

The measurements of E₂ concentrations in hens demonstrated that E₂ levels were significantly elevated ($P<0.01$), and were responsible for all significant changes observed. Fatty livers are normal for egg producing hens and occur when the increase in lipogenesis exceeds the capacity of synthesis and secretion of lipoproteins (Hermier, 1997). However, the degree to which this occurs in laying hens is modulated by many factors, both external (e.g. nutritional and environmental factors) and internal (e.g. hormones and other mediators) to the bird.

Under the influence of E₂ hens in the E₂-treated and ad libitum fed group experienced severe FLHS which was associated with an increase in mortality. Hens that did not die from FLHS were diagnosed with FLHS at necropsy. As expected, ad libitum fed hens demonstrated a higher
incidence of FLHS than hens fed a restricted feed intake diet, showing that birds with a higher feed intake, i.e. energy balance, are more at risk from FLHS than birds with a lower feed intake. In response to restricted feed intake, birds in CRF and ERF groups demonstrated a decreased BW and egg production. Decreased BW and egg production have been described in hens given a restricted feed intake diet. Overall, restricted feed intake diet given to hens treated with E₂ reduced the occurrence of FLHS, in terms of mortality and haemorrhagic score, but also affected their performance.

Treatment with E₂ induced significant liver tissue alterations, as demonstrated histologically. Normally, livers from laying hens can have some fat infiltration (microvesicular fatty change). However, hens treated with E₂ had consistent and severe infiltrations with fat and fat vacuolisations (macrovesicular fatty degeneration or steatosis). This was one of the most striking early features observed in E₂ treated hens. This study revealed transient and massive accumulation of lipids (Figure 6.11b) in the cytoplasm of hepatocytes in which a single fat vacuole distended the hepatocyte and pushed the nucleus and cytoplasm to the side. Further histological examination of the slides exposed fat droplets in the liver sinusoids (Figure 6.10b). This has not been previously reported.

In this study haematological parameters were used to follow the progression of FLHS in the hen model. Like in other animals and humans, when birds lose a quantity of blood (under 10% of their circulating blood volume) they experience a physiological stress, yet the RBC counts and HCT remain only slightly depressed (Kovach et al., 1969). Therefore, in the case of small internal haemorrhages and haematomas a simultaneous release of RBC from reserves in the bone marrow, and rapid fluid replacement or relocation across compartments occurs to maintain homeostasis (Sturkie and Griminger, 1986). In this study, RBC counts and HCT were not significantly altered, most probably due to this redistribution of the RBC and fluids which decreased the possibility to detect any changes.

The WBC counts and fibrinogen levels were altered in hens treated with E₂ when compared to controls (Table 6.3). This is the first demonstration of increased WBC counts and fibrinogen in oestrogenised birds. Hens treated with E₂ had a significant increase in leukocytes 24 h post-treatments and this number continued to increase in weeks 1, 2 and 3. Simultaneously, there was an elevation of plasma fibrinogen which correlated with WBC increases. Fibrinogen is considered a moderate acute phase protein that circulates in the plasma in response to most forms of tissue injury, infection, or inflammation (Gruys et al., 2005; Mosesson et al., 2001).
8.5 **Is inflammation involved in the pathogenesis of FLHS?**

In studies (Chapter 6) using the E\textsubscript{2} hen model there were indications that inflammation was involved in the aetiology of FLHS. To explore this possibility an E\textsubscript{2} & LPS hen model was employed (Chapter 7) and this permitted evaluation of inflammatory markers including gene expression levels of important inflammatory cytokines in hepatocytes. The cytokines targeted in this study (IL-1\textbeta, IL-6, and IL-18) are of interest because they are involved in the generation of systemic and local responses to infection and injury by generating fever, activating lymphocytes and by promoting leukocyte infiltration at sites of infection or injury (Finotto et al., 2004). As discussed in Chapter 7 the dynamics of the expression of the different cytokines was consistent with an inflammatory LPS challenge. This challenge when coupled with E\textsubscript{2} injections increased the severity of hepatic lesions. In addition, the changes in the circulating concentrations of fibrinogen (a marker of liver inflammation) (Levi et al., 2003) were consistent with cytokine modulation following an hepatic inflammatory insult (Caso et al., 2009).

It is known that an inflammatory response can be induced by bacterial toxins (LPS) which trigger liver inflammation (Kaisho and Akira, 2002) inducing local and systemic inflammation which is associated with progression of liver injury (Harte et al., 2010). Bacterial LPS has been implicated in the pathogenesis of acute liver disease through its induction of the pro-inflammatory cytokines. Many studies have supported a possible role for endotoxin in metabolic diseases. In laying hens this is an area that has received little attention and should be explored. Perhaps a gut microbiota induced metabolic endotoxemia and resulting inflammation is involved in the development of FLHS.

Although it was not possible to separate out the relative effects of E\textsubscript{2} and LPS from the current studies, the results suggest a role for inflammation in the pathogenesis of FLHS. Maybe in the field where commercial laying hens have high circulating concentrations of oestrogen, sporadic exposures to inflammatory challenge is sufficient to precipitate FLHS in birds with pre-existing fatty livers.

8.6 **Implications of the study**

Studies conducted in this thesis indicate that FLHS is a significant disease of caged layer hens in Queensland and impacts on hen health and welfare. Significant economic losses to producers occur because egg production drops and mortality increases. The results arising from this research pose as many questions as it answers. From this research and the literature, FLHS is a single disease with multiple aetiologies and this has a number of implications for both industry and further research.
With regard to industry:

- Egg producers should be made aware of the presence of this syndrome in laying flocks and its significant impact on egg production and hen mortality.
- Egg producers should be advised of the importance of monitoring their flocks for FLHS by systematic necropsy of dead hens. Monitoring BW, especially in high producing flocks and heavy layer strains, may assist in identifying flocks predisposed to FLHS.

From a research perspective, the apparent role of both inflammation and elevated circulating oestrogen concentrations in inducing FLHS is a useful step in understanding the pathogenesis of this condition. This model should prove useful in further studies of the disease, including:

- The development of non-invasive techniques to detect FLHS in commercial laying flocks. This will assist egg producers to detect FLHS and make important management decisions in relation to this metabolic disease while maximising egg production efficiency.
- Further exploration of the interactions between metabolism, inflammation and endocrinology in the pathogenesis of FLHS, especially the effects of inflammatory factors on liver cells and the occurrence of the condition. This would help explain why only some laying hens develop FLHS while all have fatty livers.

Finally, it will not be possible to develop strategies to reduce the incidence of FLHS until the factors that predispose birds to the condition are fully understood.
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Appendices

Appendix 1

Diet specifications (feed analysis for Farm 1, 2, and 3)

CONFIDENTIAL

Table 1  Farm 1 feed analysis

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Amount</th>
<th>Units</th>
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</thead>
<tbody>
<tr>
<td>Volume %</td>
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</tr>
<tr>
<td>Na %</td>
<td>0.139</td>
<td></td>
</tr>
<tr>
<td>Protein %</td>
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<td>K %</td>
</tr>
<tr>
<td>Fat %</td>
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<td>CI %</td>
</tr>
<tr>
<td>Fibre %</td>
<td>2.40</td>
<td>Methionine %</td>
</tr>
<tr>
<td>ME_POUL MJ MJ/kg</td>
<td>11.40</td>
<td>METH + CYST %</td>
</tr>
<tr>
<td>Calcium %</td>
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<td>Linoleic</td>
</tr>
<tr>
<td>Phosphorus %</td>
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<td>Choline</td>
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<td>AV. Phosphorus %</td>
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<td>Soy+FF</td>
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<td>CAL:PHOS</td>
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Table 2  Farm 2 feed analysis

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<tr>
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</thead>
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<tr>
<td>PROTEIN</td>
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</tr>
<tr>
<td>Fibre</td>
<td>4.3</td>
<td>%</td>
</tr>
<tr>
<td>FAT</td>
<td>5.6</td>
<td>%</td>
</tr>
<tr>
<td>CALCIUM</td>
<td>3.82</td>
<td>%</td>
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<tr>
<td>PHOSPHORUS</td>
<td>0.83</td>
<td>%</td>
</tr>
<tr>
<td>M.E.Poultry MJ</td>
<td>11.6</td>
<td>MJ/kg</td>
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</table>
### Table 3  Farm 2 Production formula: Layer 120

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<th>Nutrient Name</th>
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<tr>
<td>PROTEIN</td>
<td>16.69</td>
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<tr>
<td>FAT</td>
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<tr>
<td>AVAIL. PHOSPHORUS</td>
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<td>%</td>
</tr>
<tr>
<td>PHYTATE PHOSPHORUS</td>
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<td>%</td>
</tr>
<tr>
<td>METHIONINE</td>
<td>0.435</td>
<td>%</td>
</tr>
<tr>
<td>METHIONINE + CYSTINE</td>
<td>0.700</td>
<td>%</td>
</tr>
<tr>
<td>M.E.Poultry-MJ</td>
<td>11.50</td>
<td>MJ/kg</td>
</tr>
<tr>
<td>CHOLINE</td>
<td>1,300</td>
<td>mg/kg</td>
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<tr>
<td>DIGEST LYSINE</td>
<td>0.669</td>
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</tr>
<tr>
<td>SODIUM</td>
<td>0.180</td>
<td>%</td>
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<td>POTASSIUM</td>
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<td>%</td>
</tr>
<tr>
<td>CHLORIDE</td>
<td>0.200</td>
<td>%</td>
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Table 4  Comparison of laying nutrient levels as recommended by breeders until 44 wk of age (Australian ingredients) and provided in the diets used in farms surveyed

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Hy-Line&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Farm 1 (farm-mixed)</th>
<th>Farm 2 (farm-mixed)</th>
<th>ISA Brown&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Farm 3 (commercial)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sorghum, wheat and soybean based meal</td>
<td>Corn based meal (100 g)</td>
<td>Sorghum, wheat, soybean, and meat based meal</td>
<td></td>
</tr>
<tr>
<td>Metabolisable Energy (MJ/kg)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>11.7</td>
<td>11.4</td>
<td>11.6</td>
<td>11.5-11.8</td>
<td>11.5</td>
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<tr>
<td>Protein (g/kg)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>16.5</td>
<td>17.3</td>
<td>19.0</td>
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<td>16.7</td>
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<tr>
<td>Fibre</td>
<td>2.50</td>
<td>4.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat (g/kg)</td>
<td>3.91</td>
<td>5.60</td>
<td></td>
<td></td>
<td>6.14</td>
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<tr>
<td>Calcium (g/kg)</td>
<td>3.50</td>
<td>3.90</td>
<td>3.82</td>
<td>4.1-4.3</td>
<td>3.80</td>
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<tr>
<td>Av. Phosphorus (g/kg)</td>
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<td>0.38</td>
<td>0.48</td>
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<td>0.48</td>
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<td>Methionine (%)</td>
<td>0.44</td>
<td>0.32</td>
<td></td>
<td>0.41</td>
<td>0.44</td>
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<tr>
<td>Methionine + Cystein (%)</td>
<td>0.74</td>
<td>0.61</td>
<td></td>
<td>0.71</td>
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<td>Digest. Lysine (%)</td>
<td>0.84</td>
<td>0.80</td>
<td></td>
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<td>0.67</td>
</tr>
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</table>

<sup>1</sup>Brown-egg-layers at 110 g of feed per hen daily

<sup>2</sup>ISA Brown at 110 g of feed per hen daily from 28 wks to the end of lay;

<sup>3</sup>Energy required per hen per day in relation to BW (average of 1750 g) and rate of egg production (80%)

<sup>4</sup>Derived with corn-soybean meal diet
Appendix 2

Table 5  Composition of laying hen diet used for trial at Gatton Layer facility

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Volume %</th>
<th>CAL:PHOS</th>
<th>5.95</th>
<th>Threonine</th>
<th>0.59</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein %</td>
<td>17.30</td>
<td>CAL:AVPHOS</td>
<td>10.29</td>
<td>Leucine</td>
<td>1.47</td>
</tr>
<tr>
<td>Fat %</td>
<td>3.91</td>
<td>Na %</td>
<td>0.14</td>
<td>Isoleucine</td>
<td>0.68</td>
</tr>
<tr>
<td>Fiber %</td>
<td>2.49</td>
<td>K %</td>
<td>0.53</td>
<td>Tryptophan</td>
<td>0.18</td>
</tr>
<tr>
<td>ME_PO Kcal kcal/kg</td>
<td>270.97</td>
<td>Cl %</td>
<td>0.174</td>
<td>Avlys_poult</td>
<td>0.67</td>
</tr>
<tr>
<td>ME_POUL MJ MJ/kg</td>
<td>11.39</td>
<td>Na + K_Cl meq/kg</td>
<td>147.96</td>
<td>Linoleic</td>
<td>1.21</td>
</tr>
<tr>
<td>Calcium %</td>
<td>3.91</td>
<td>LYSINE %</td>
<td>0.78</td>
<td>Choline</td>
<td>878.42</td>
</tr>
<tr>
<td>Phosphorus %</td>
<td>0.66</td>
<td>METHIONONE %</td>
<td>0.32</td>
<td>Legumes</td>
<td>0.0</td>
</tr>
<tr>
<td>AV Phos %</td>
<td>0.38</td>
<td>METH + CYST%</td>
<td>0.61</td>
<td>Soy+FF</td>
<td>10.93</td>
</tr>
</tbody>
</table>
Appendix 3

Necropsy record sheet

<table>
<thead>
<tr>
<th>Owner's identification:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bird identification:</td>
<td></td>
</tr>
<tr>
<td>Breed/Age:</td>
<td></td>
</tr>
<tr>
<td>Weight:</td>
<td></td>
</tr>
<tr>
<td>Date/Time of possible death:</td>
<td></td>
</tr>
<tr>
<td>Cause of death: found dead/killed/injured etc.</td>
<td></td>
</tr>
<tr>
<td>Relevant clinical signs prior to death:</td>
<td></td>
</tr>
<tr>
<td>Date of examination:</td>
<td>Preservation of body: fresh/frozen</td>
</tr>
</tbody>
</table>

1. External examination

<table>
<thead>
<tr>
<th>Normal (Yes/No)</th>
<th>Describe abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td></td>
</tr>
<tr>
<td>Feather/Comb condition</td>
<td></td>
</tr>
<tr>
<td>External parasites</td>
<td></td>
</tr>
<tr>
<td>Eyes/Ears/Nose</td>
<td></td>
</tr>
<tr>
<td>Beak/Oral cavity</td>
<td></td>
</tr>
<tr>
<td>Foot condition</td>
<td></td>
</tr>
<tr>
<td>General carcass condition</td>
<td></td>
</tr>
</tbody>
</table>

2. Examination of the organs/tissues/contents

<table>
<thead>
<tr>
<th>Normal (Yes/No)</th>
<th>Describe abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td></td>
</tr>
<tr>
<td>Musculoskeletal</td>
<td></td>
</tr>
<tr>
<td>Peritoneal / Pleural cavities</td>
<td></td>
</tr>
<tr>
<td>Oesophagus</td>
<td></td>
</tr>
<tr>
<td>Crop</td>
<td></td>
</tr>
<tr>
<td>Proventriculus</td>
<td></td>
</tr>
<tr>
<td>Gizzard</td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td></td>
</tr>
<tr>
<td>Cloaca</td>
<td></td>
</tr>
<tr>
<td>Liver / Pancreas</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>Thymus (if present)</td>
<td></td>
</tr>
<tr>
<td>Bursa (if present)</td>
<td></td>
</tr>
<tr>
<td>Air sacs/Lungs/Trachea</td>
<td></td>
</tr>
<tr>
<td>Heart/circulatory</td>
<td></td>
</tr>
<tr>
<td>Nervous system</td>
<td></td>
</tr>
<tr>
<td>Genital/Ovary/Oviduct</td>
<td></td>
</tr>
<tr>
<td>Kidney/ureter/adrenal gland</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
</tbody>
</table>

3. Further records
a. Sample collection: Yes / No
   i. Organ
   ii. Tissue
   iii. Content (fluid)
   iv. Culture (swabs)

b. Smear preparation:

c. Pictures:

d. Other:

4. Ancillary diagnostics:
   a. Histopathology ( )
   b. Toxicology ( )
   c. Cytology ( )
   d. Parasitology ( )
   e. Microbiology ( )
   f. Virology ( )
   g. Other ( )

5. Disposal arrangements:

6. Tentative diagnosis/comments:

7. Examiner:
Appendix 4

Questionnaire used to survey farmers in Queensland

Questionnaire

Fatty Liver Haemorrhagic Syndrome (FLHS) Survey

Farm Name/Address:

Your participation in this survey will be much appreciated and ensure results which will benefit your organisation and the industry as a whole. Please be assured that you will not be identified individually in the survey outcomes. Your confidentiality is respected.

For further information please contact: A. Shini 07 5460 1159 or email a.shini@uq.edu.au.

Please return the questionnaire as an email attachment, or fax (07) 5460 1444, or send by mail to: A. Shini, School of Animal Studies, University of Queensland, Gatton QLD 4343.

1. GENERAL
   a) Length of time poultry farm has been operational: _______ years
   b) Average number of caged layers each year / laying cycle: ___________
   c) Housed in (number of sheds) _______________
   d) N of flocks currently _________ Age of flock _________________

   If flocks have different age:
      1:
      2:
      3:

2. HOUSING
   a) Cage type ______________________________________________________:

      With environmentally controlled ventilation ☐ naturally ventilated ☐ no ventilation ☐

      Size of cages ________________

      Number of hens per cage ________

   b) Shed temperature ______-____°C
3. FEED AND FEED INGREDIENTS/ANALYSIS

What kind of feed is used?: Commercial □ Self-prepared □

a) Feed Formulation Information
Please fill in ingredients/feed analysis or attach a list of the diet:

<table>
<thead>
<tr>
<th>Feed Type</th>
<th>Pre-Lay Diet</th>
<th>Phase 1 Layer Diet</th>
<th>Phase 2 Layer Diet</th>
<th>Phase 3 Layer Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (week)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy (MJ/KG) or (Kcal/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibre (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium (g/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avail. Phosphorus (g/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca:P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine + Cystein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b) Are there any other chemical components in the feed? yes □ no □

c) If yes, what? ________________________________________________________________

4. HENS AND PERFORMANCE

a) What breed and/or strain do you currently use for your flock? ______________________

b) Are hens reared on farm? yes □ no □
   If yes, how are hens reared? Floor □ Cage (Wire) □ Barn □

c) At what age are pullets placed in cages? __________________________________________

d) At what age do you dispose of hens? _____________________________________________

e) Are hens replaced on an ‘all in, all out’ basis? _________________________________

f) Do you use a lighting regime? yes □ no □ If yes, what is it? ______________________

g) What is your average rate of production? ________________________________________
h) What is your rate of production at peak? ______________________________________

i) What is your rate of production at this time of laying cycle? ______________________

j) What is your average rate of mortality? ______________________________________

k) What is your average rate of mortality at this time of laying cycle? ________________

5. MANAGEMENT

a) Are birds beak trimmed? yes □ no □ If yes, at what age? ______________________

b) Do you undertake a regular weighing programme? yes □ no □ If yes, how often?

c) Do you undertake a regular worming/external parasite eradication programme?

yes □ no □ If yes, how often? weekly □ monthly □ tri-monthly □ yearly □ as required □

d) Against which diseases are your flocks vaccinated? (please tick)

<table>
<thead>
<tr>
<th>Disease</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IBV (infectious bronchitis)</td>
<td></td>
</tr>
<tr>
<td>ILT (infectious laryngotracheitis)</td>
<td></td>
</tr>
<tr>
<td>Marek’s disease</td>
<td></td>
</tr>
<tr>
<td>Newcastle disease</td>
<td></td>
</tr>
<tr>
<td>Fowl pox</td>
<td></td>
</tr>
<tr>
<td>Coccidiosis</td>
<td></td>
</tr>
<tr>
<td>Infectious coryza</td>
<td></td>
</tr>
<tr>
<td>MG (Mycoplasma gallisepticum)</td>
<td></td>
</tr>
<tr>
<td>MS (Mycoplasma synoviae)</td>
<td></td>
</tr>
<tr>
<td>AE (avian encephalomyelitis)</td>
<td></td>
</tr>
<tr>
<td>EDS (inactivated egg drop syndrome)</td>
<td></td>
</tr>
</tbody>
</table>
What were the main causes of mortality (birds found dead and culled) on your farm (if known)?

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of mortalities</th>
<th>Age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fowl cholera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marek’s disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Salmonella sp.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tracheitis (<em>Mycoplasma sp.</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coccidiosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spotty liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty liver haemorrhagic syndrome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg peritonitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ingluvitis (inflammation of the crop)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salpingitis (inflammation of the oviduct)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prolapse/protrusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cannibalism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical injury (i.e. Broken leg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat Stress</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Who determines the cause of mortality?

<table>
<thead>
<tr>
<th>Person</th>
<th>Percentage of time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Owner/Manager</td>
<td></td>
</tr>
<tr>
<td>Veterinarian</td>
<td></td>
</tr>
<tr>
<td>Pathology Lab</td>
<td></td>
</tr>
</tbody>
</table>

Thank you for your cooperation!
### Table 6  Statistical analysis showing results from unpaired Student t test (p-value)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Treatment</th>
<th>Difference from control</th>
<th>SE (difference)</th>
<th>T (statistic)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1β</strong></td>
<td><strong>CONT 3 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>1.094</td>
<td>0.4129</td>
<td>0.2283</td>
<td>0.831</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>1.884</td>
<td>0.3348</td>
<td>3.2381</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td><strong>CONT 24 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>3.086</td>
<td>0.4287</td>
<td>7.1989</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>4.842</td>
<td>0.2424</td>
<td>19.9738</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>LPS &amp; E2</td>
<td>1.379</td>
<td>0.1848</td>
<td>7.4637</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td><strong>CONT 3 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>5.483</td>
<td>0.6857</td>
<td>7.9958</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>5.182</td>
<td>0.5574</td>
<td>9.2965</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td><strong>CONT 24 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>2.775</td>
<td>0.0670</td>
<td>41.3842</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>1.019</td>
<td>0.1742</td>
<td>5.8478</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>LPS &amp; E2</td>
<td>3.483</td>
<td>0.0535</td>
<td>65.1614</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td><strong>IL-18</strong></td>
<td><strong>CONT 3 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>1.286</td>
<td>3.6318</td>
<td>0.3542</td>
<td>0.741</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>2.039</td>
<td>2.9579</td>
<td>0.6895</td>
<td>0.528</td>
<td></td>
</tr>
<tr>
<td><strong>CONT 24 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>-1.090</td>
<td>3.1212</td>
<td>0.3492</td>
<td>0.745</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>2.076</td>
<td>3.3374</td>
<td>0.6222</td>
<td>0.568</td>
<td></td>
</tr>
<tr>
<td>LPS &amp; E2</td>
<td>2.481</td>
<td>3.6205</td>
<td>0.6854</td>
<td>0.531</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)E2 = oestrogen-treated; LPS = LPS-treated; E2 & LPS-treated; Oil = corn-oil treated; Saline = PBS-treated; CONT = control (no treatment); P evaluating the hypothesis that the difference is zero
Appendix 6

a) E2 treated ad libitum fed birds

b) E2 treated restricted feed intake birds

Figure 2  Distribution of individual values of oestradiol for birds administered with exogenous E2
### Table 7  Plasma concentration of GGT, CHOL, GLU, and TP in treated and control hens

<table>
<thead>
<tr>
<th></th>
<th>GGT U/L</th>
<th>CHOL mmol/L</th>
<th>GLU mmol/L</th>
<th>TP g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 3 h</td>
<td>23.7</td>
<td>2.7</td>
<td>10.5</td>
<td>55.1</td>
</tr>
<tr>
<td>Control 24 h</td>
<td>25.2</td>
<td>2.5</td>
<td>11.6</td>
<td>47.9</td>
</tr>
<tr>
<td>Control 1 wk</td>
<td>24.5</td>
<td>2.8</td>
<td>11.3</td>
<td>56.3</td>
</tr>
<tr>
<td>E2 3 h</td>
<td>33.7</td>
<td>4.6</td>
<td>10.3</td>
<td>54.7</td>
</tr>
<tr>
<td>E2 24 h</td>
<td>30.5</td>
<td>3.8</td>
<td>10.7</td>
<td>51.6</td>
</tr>
<tr>
<td>E2 1 wk</td>
<td>28.8</td>
<td>3.7</td>
<td>11.6</td>
<td>57.3</td>
</tr>
<tr>
<td>LPS 3 h</td>
<td>34.3</td>
<td>2.8</td>
<td>11.2</td>
<td>49.0</td>
</tr>
<tr>
<td>LPS 24 h</td>
<td>25.3</td>
<td>1.8</td>
<td>11.7</td>
<td>50.8</td>
</tr>
<tr>
<td>LPS 1 wk</td>
<td>26.2</td>
<td>1.8</td>
<td>11.5</td>
<td>56.0</td>
</tr>
<tr>
<td>E2&amp;LPS 3 h</td>
<td>30.8</td>
<td>3.4</td>
<td>10.9</td>
<td>45.0</td>
</tr>
<tr>
<td>E2&amp;LPS 24 h</td>
<td>27.2</td>
<td>3.5</td>
<td>10.5</td>
<td>48.3</td>
</tr>
<tr>
<td>E2&amp;LPS 1 wk</td>
<td>28.5</td>
<td>3.7</td>
<td>10.2</td>
<td>45.7</td>
</tr>
</tbody>
</table>