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Variation in performance traits of laying hens and the relationships with egg quality and egg safety

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Declaration

I declare that this thesis has never been submitted for any degree at the University of Nottingham, nor at any other university. I declare that the studies contained in this thesis are original and mine.

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Acknowledgements and dedication

‘A supervisor is one who gives you space to fall, gives you time and support you to rise, and then gives you a place when you rise – Doreen Anene’.

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Abstract

The feed consumption, body weight, egg weight, and rate of lay of the hen, as well as the albumen, yolk, and shell characteristics of the egg, are important measures of productivity, quality, and safety in the layer industry. However, in the literature, there is little information on the onset and extent of variation of these traits among individual hens, the persistence of these differences during the laying period and how these variations are related to egg quality and safety as well as hen health and welfare. The overarching goal of this thesis was to control environmental variables such as food and housing conditions and determine the variation in hen performance, egg quality and organ characteristics between individual birds. This research also aimed to explore the effect of varying feed efficiency on hen body weight, egg production, egg quality, egg safety and organ characteristics from early lay to late laying stages. Finally, this research evaluated a dietary intervention to improve uniformity of hen and egg traits. To achieve these objectives, the first study investigated the inherent variation and associations in hen performance traits (feed intake, egg production, and body weight changes) and egg quality measurements (albumen, yolk, and shell characteristics) of individually housed hens in the early stages of lay. The second study in this thesis evaluated the effect of varying feed efficiencies on body weight changes, egg production, nutrient digestibility, and egg quality characteristics of individual ISA Brown hens in the mid laying stage of lay. The third study sought to estimate the effect of varying feed efficiency and body weight on organ characteristics and incidences of FLHS in mid laying hens. The fourth study examined the differences in the bacteriostatic activity and proteome composition of egg albumen from ISA Brown hens of varying FE while the final study evaluated the effect of temporary or permanent quantitative feed restriction beginning from point of lay, on performance traits, egg quality indices, organ characteristics and occurrences of fatty liver haemorrhagic syndrome of individually housed hens. Summarily, a total of 455 pullets were

individually housed and monitored for performance and egg quality differences from 25 to 30 weeks of age. At 30 weeks old, the hens were ranked based on feed efficiency into high feed efficiency (HFE), medium feed efficiency (MFE) and low feed efficiency (LFE) groups, and then monitored for performance, egg quality and organ characteristics until the late stages of lay. The results from this research showed that important variations in hen and egg traits exists and these variations are present from the initial stages of lay. Hens maintained the feed efficiency ranking which they attained in early lay until at least 75 weeks of age. The HFE hen cohort consumed lesser feed and had a lower bodyweight compared to the hens from the LFE and MFE group. Interestingly, FE ranking did not have an effect on egg production up to 75 weeks. Hens ranked as LFE had higher abdominal fat weight, fatty livers, and higher fatty liver haemorrhagic syndrome (FLHS) lesion scores, compared to hens ranked as HFE. Eggs collected from hens ranked as HFE had a higher albumen quality and a stronger bacteriostatic activity against gram-negative *Salmonella typhimurium* and gram-positive *Staphylococcus aureus*, compared to the LFE groups. Proteomics profiling detected an additional 20 protein groups in albumen from HFE hens which were not found in albumen from LFE hens. There were no differences in the shell characteristics of eggs across all FE groups from 30 weeks up to 75 weeks. A quantitative feed restriction intervention study to improve uniformity of performance traits and egg quality was conducted. A total of 150 hens, placed into three feeding treatments - ad libitum feeding (ALF, from 22 to 46 weeks), temporary restricted (TRF, restricted until 40 weeks and transitioned to ALF status) and permanently restricted (PRF, restricted from 22 to 46 weeks), (n = 50 per treatment), were monitored from 22 weeks to 46 weeks of age. Hens on the TRF treatment showed compensatory feeding and body weight change increase when transitioned to ALF status. Although restricting feed consumption until 40 weeks of age did not control further feed intake and body weight gain, hens on the PRF treatment maintained a more uniform body

weight, had better albumen quality, and had a lower fatty liver haemorrhagic lesion score compared to hens on the ALF treatment. This research showed that among individually housed ISA Brown hens, variation exists in hen traits which set in from the early stages of lay. These differences are associated with albumen quality, egg abnormality, abdominal fat deposition and incidences of fatty liver haemorrhagic syndrome. The findings of this study recommends that hen body weight should be controlled to around the breed exception from early lay as this has lifelong effects on the performance and health of the bird.

Keywords: individual laying hens, feed efficiency, egg quality, organ characteristics, body weight, variation.

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List of Abbreviations

ADFI	Average Daily Feed Intake
ALF	<i>Ad libitum</i> Feeding
CV	Coefficient of Variation
EM	Egg Mass
EP	Egg Production
EW	Egg Weight
FBW	Final Body Weight
FCR	Feed Conversion Ratio
FE	Feed Efficiency
FLHS	Fatty Liver Haemorrhagic Syndrome
HFE	High Feed Efficiency
HU	Haugh unit
IBW	Initial Body Weight
LFE	Low Feed Efficiency
MFE	Medium Feed Efficiency
PRF	Permanent Restricted Feeding
SD	Standard Deviation
SEM	Standard Error of Mean
STEM	Science, Technology, Engineering and Mathematics
SW	Slaughter Weight
TBARS	Thiobarbituric Acid Reactive Substances
TRF	Temporary Restricted Feeding
WOA	Weeks of Age

CHAPTER ONE

1. Literature Review

1.1 Global trends in egg production and consumption

1.1.1 – Egg consumption and production

The layer industry is an important part of the poultry sector and eggs, the key marketable product of hens, has been recognized and accepted as an excellent and inexpensive source of food (WHO 2002; Guyonnet 2012). Due to various reasons including demographics, urbanisation, wealth status and income growth, global egg consumption has increased over the years from 35 million metric tonnes in 1990 to 86 million metric tonnes in 2020 (Shahbandeh, 2022), and this is expected to be doubled by 2050. In Australia, egg consumption grew from 221 eggs per person in 2014/2015 to 249 eggs in 2020/2021, with an average egg weighing 58 grams (Australian Eggs, 2022). In the United Kingdom, the overall per capita consumption has grown from 171 eggs per person per year to 202 eggs per person per year from 2004 to 2021 respectively (Egg Info UK, 2021). In Asia, egg consumption is estimated at 21.2 kg per person in Japan (about 337 eggs), and 15.8 kg per year per person (254.8 eggs) in China (International Egg Commission, 2019). Due to outbreak of the avian flu and the rising cost of egg prices in Mexico, consumption of eggs declined from 22.0 dozens per capita in 1980 to 17.1 dozens per capita in 1995 (Sawe, 2018). However, due to a variety of factors including the introduction of designer eggs with omega-3 fatty acids in 1996, a gradual increase to 20.5 dozens per capita in 2012 was observed (Zaheer, 2015). A summary of the average production, consumption, and self-sufficiency ratio of 35 countries as reported by the International Egg Commission in 2019, is presented in Table 1.1.

Table 1.1 The production, consumption and self-sufficiency rate of shelled eggs and egg products (liquid and dried egg products) of various countries across the world.

Country	Production ‡ (Tonnes)	Consumption ‡ (eggs/person/year)	Self Sufficiency rate %	Human Population (Million)
Argentina	1,061,524	271	103	44.5
Australia	361,980	244	-	25.1
Austria	116,174	236	87	8.8
Brazil	2,669,242	212	-	201
Canada	692,664	253	91	37.1
China	22,263,000	254.8	100	1395
Columbia	876,529	293	100	49.8
Cyprus	8,938	205.9	83.7	0.9
Denmark	67,500	248	85	5.7
Finland	76,100	198	115	5.52
France	1,182,000	213	100	66.9
Germany	846,000	235	69.4	82.78
Hungary	85,837	238	88	9.78
India	5,712,400	76	98	1358
Iran	964,550	183	99	82.8
Ireland	53,096	188	92	4.8
Italy	772,950	208	97.3	60.4
Japan	3,147,058	337	96	127
Kazakhstan	307,720	254	100	18.4
Mexico	2,922,607	367.7	99	123.9

Netherlands	638,000	205	300	17
New Zealand	62,757*	237	100	4.9
Peru	466,000	224	100	33
Poland	592,000	141	160	38.3
Portugal	142,505	152.8	110.3	10.3
Russia	271,200	307	110	146.8
Slovakia	177,744	228	93	5.5
South Africa	433,880	129.7	101	57.7
Spain	932,000	260	107	46.7
Sweden	11,171	235	95	10
Switzerland	71,316	180.7	63	8.6
Turkey	1,576,050	224	127	82
United Kingdom	777,437	199	87	66.2
USA	7,020,942	284	103	327

Source: (International Egg Commission, 2019), (FAOSTAT, 2019)*. † Shell eggs and egg product (liquid and dried egg products).

Despite the outstanding nutrient profile of the hen egg, they remain scarcely consumed in many low-income settings. In Kenya, Nigeria and South Africa, based on an average egg weight of 50 g, the per capita egg consumption was capped at 37, 69 and 130 eggs per person per year respectively (GAIN, 2019). This extremely low consumption rates could be as a result of high cost of production inputs particularly feed, inefficient production systems as well as cultural beliefs and taboos surrounding egg consumption (Tukur, 2011; GAIN, 2019). It is noteworthy that the average consumption of protein of animal origin (g/capita/day) in Africa and Asia, has remained below the global recommended standard of 31.1 g per capita

per day (Figure 1.1), while the prevalence of severe food insecurity in Africa and Latin America and the Caribbeans (LAC) was higher than the global threshold of 8.1% and 11.9% in 2015 and 2020 respectively (Figure 1.2).

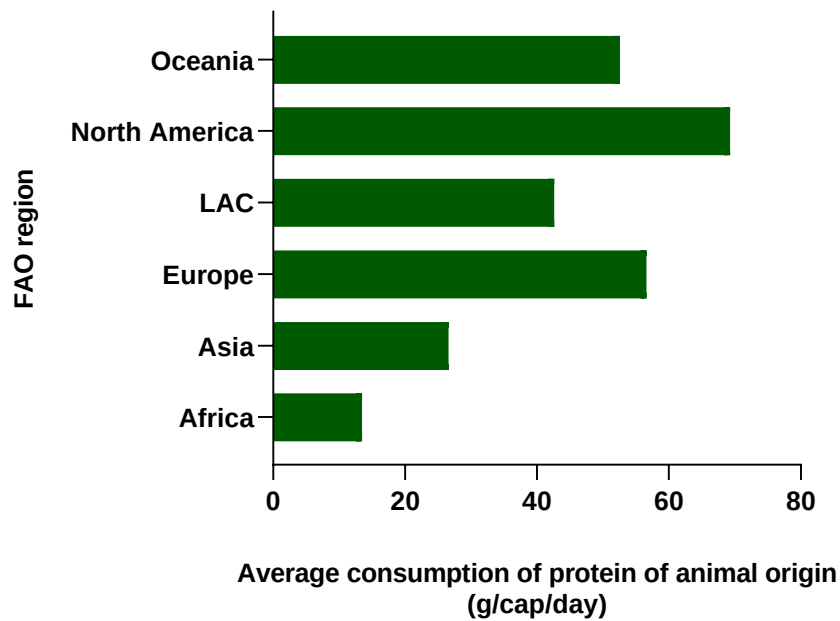


Figure 1.1 - The average consumption of protein of animal origin, g/capita/day across various FAO Regions. LAC – Latin America and Caribbeans. Source (FAOSTAT, 2022)

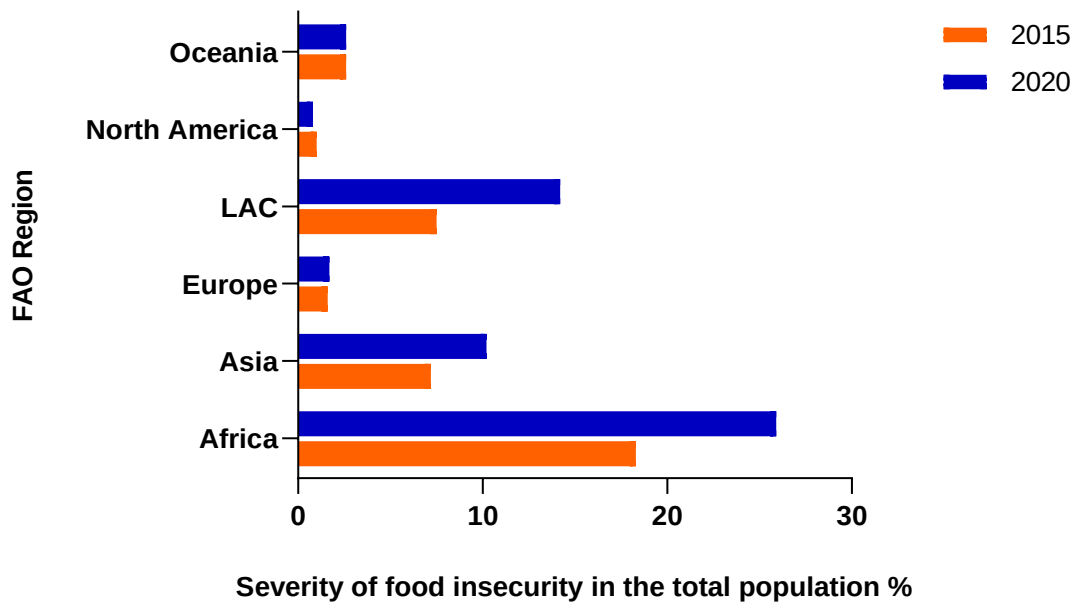


Figure 1.2 - The percentage prevalence of severe food insecurity in the total population across various FAO regions. LAC – Latin America and Caribbeans. Source (FAOSTAT, 2022).

To meet the growing demand of animal protein consumption, egg production has increased by about 500% in the last four decades from 14.4 million tonnes in 1961 to 72.1 million tonnes in 2015. This has also increased further by 20% to 86.7 million tonnes in 2020 (FAOSTAT, 2015, 2020), and is projected to reach 120 million tonnes by 2030. The contribution of the various Food and Agricultural Organisation Regions to global egg production is presented in Figure 1.3. Asia has remained the world's largest producer of shelled eggs contributing 43 billion tonnes and 54 billion tonnes representing 59.7% and 62.4% of global egg production in 2015 and 2020 respectively. Africa and Oceania (including New Zealand, Australia, Polynesia, Micronesia) contributed the least towards global egg output - 4.6% and 4.1% for Africa and 0.4% and 0.38% for Oceania in 2015 and 2020 respectively.

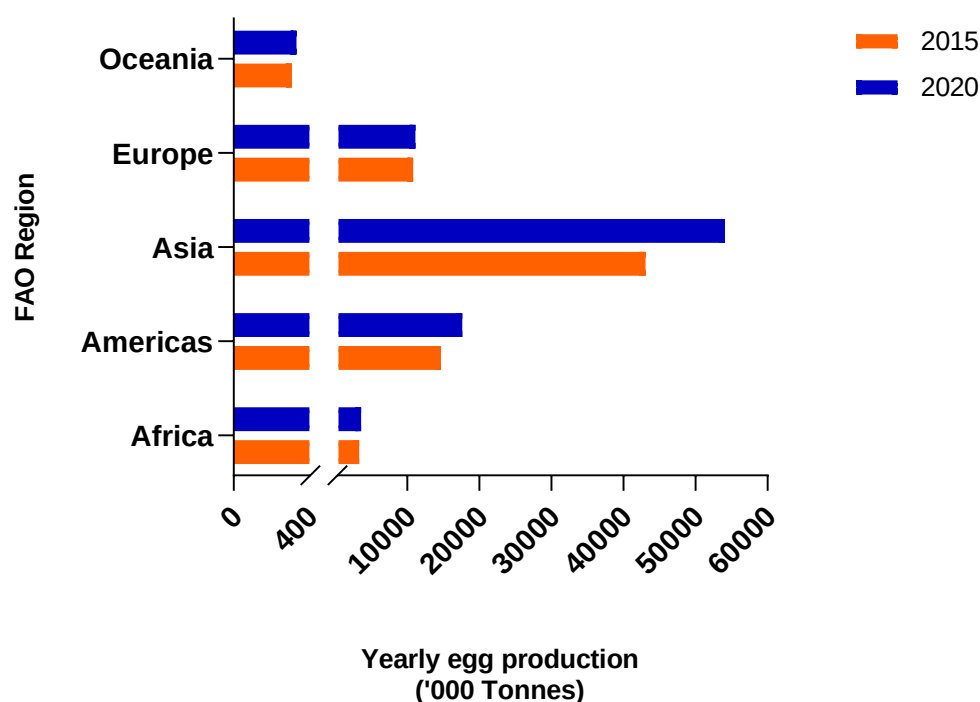


Figure 1.3 - Contribution of the various FAO Regions towards global egg production in 2015 and 2020. Source: (FAOSTAT, 2015, 2020). Aggregate may include official, semi-official, estimated, or calculated data. FAO – Food and Agricultural Organisation.

In Europe, the priority is to increase egg production, stabilise egg quality, improve persistency in lay and prolong the laying cycle up to 90 –100 weeks. Increasing the laying life of commercial hens up to 100 weeks might face some scrutiny, however, it presents a potential for more efficient utilisation of financial and environmental resources. For example, a review from Bain et al (2016), estimates that extending egg production up to 100 weeks can lead to an additional 25 more eggs per hen, which would increase output, but potentially reduce the numbers of breeding and commercial hens by up to 2.5 million birds per annum thereby improving the carbon foot print of egg production.

1.1.2 – Hen production housing systems

Housing system in commercial egg production enterprises is an important management factor which has faced major scrutiny, especially in the recent decades. In 1999, with exceptions of farms with fewer than 350 laying hens or farms rearing breeder hens, the European Union directed the ban of conventional battery cage system due to concerns about hen welfare, beginning from 2012 (Council Directive 1999/74/EC). In this directive, cages should only be used when enriched with nests, perches, dust baths and other facilities that allow for the natural behaviour of the bird. By simple definitions, in cage system, hens are housed in climate-controlled sheds either singly or in a colony of 4 – 8. They are sometimes referred to as battery cages and are usually set up in multi-tiered cages, sometimes enriched or not. Feed and water are provided in each battery cage and eggs when laid rolls out and are collected in front of the cage, while excreta are collected on the manure belt below the wire mesh flooring and disposed. In enriched cage housing, nesting boxes, and perches are provided. Barn housing are also climate-controlled sheds but without cages. The birds are able roam freely within the shed with no encounter with the external environment. Feed, water, nesting boxes and other enrichments are provided at designated points across the barn, while excreta is collected through the deep litter system. Hens raised on a free-range system have access to an outdoor range during the day but are housed securely and comfortably in sheds at night. Nesting boxes and feed are provided at designated areas in the shed and on the range (Australian Eggs, 2022).

A report from International Egg Commission (2019) however shows that 68% of egg production was done in cage/ colony system while 17.1%, 12% and 4.2% of production involved barn, free range and organic systems respectively, as presented in Figure 1.4. The country breakdown of housing system of the 32 countries reviewed is shown in Table 1.2.

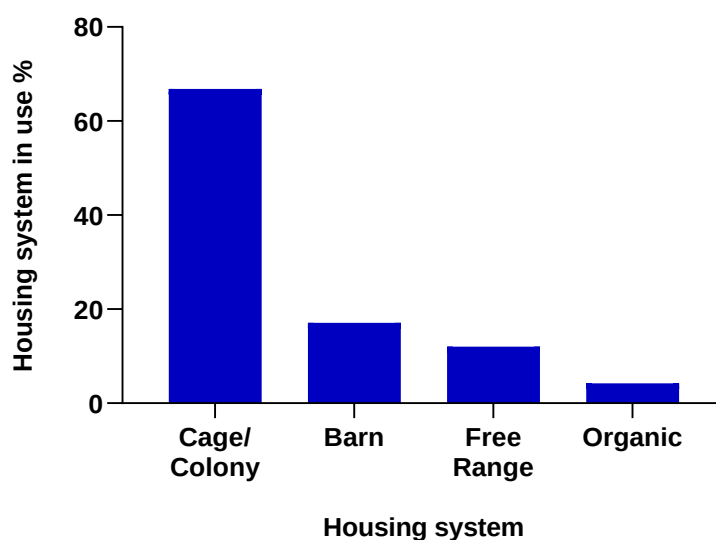


Figure 1.4 - Average housing systems used in egg production across 32 countries in the world. Source (International Egg Commission, 2019).

Table 1.2 - Housing systems used in egg production from 32 countries in the world.

Country	Cage or colony	Barn	Free range	Organic
Argentina	89	8	3	0
Australia	54.5	9.1	36.4	0
Austria	0.8	64.4	23.5	11.7
Canada	87	9	1	3
China	97	0	3	0
Columbia	70	30	0	0
Cyprus	71.3	15.1	11.4	2.2
Denmark	16	46	8	30
Finland	58	33	2	7
France	61	8	21	10
Germany	6.7	62	19.1	11.5

Hungary	80.8	17.4	1.4	0.5
India	100	0	0	0
Iran	100	0	0	0
Ireland	53	1	43	3
Italy	54.5	35.3	4.6	5.6
Japan	94.6	4.5	0.9	0
Kazakhstan	85	0	15	0
Mexico	99.8	0.2	0	0
Netherlands	11	61	20	8
New Zealand	66.4	3	29.6	1
Peru	93	5	1	1
Poland	88.3	9.1	2.4	0.2
Portugal	90.5	6.5	2.5	0.5
Russia	99.9	0.1	0	0
Slovakia	83	16	0.5	0.5
South Africa	86	0	14	0
Spain	83	9	7	1
Sweden	8	72	4	16
Switzerland	0	22.2	60.5	17.3
Turkey	88	3	8	1
United Kingdom	44.2	1.4	51.9	2.5
USA	83.4	11.9	0	4.7

Source (International Egg Commission, 2019).

The choice of housing for egg laying birds is an important management decision as it can impact on the bone and skeletal health of the hen, incidences of pest and parasite load, disease and biosecurity, social interaction and natural behaviours, hen performance and productivity, internal egg quality, production economics and the environment. The slow adoption of the non-cage rearing system might be as a result of the high cost of transitioning and also as a result of the inconsistent results from assessments of various housing systems as it affects hen performance and egg quality indices.

1.1.2.1 – Bone health

Hybrid hens have been selected and developed for outstanding egg productivity, however the high demand of calcium needed for daily egg production places a demand on skeletal calcium reserves and structural integrity which is a crucial factor for health, welfare, optimum productivity, and egg quality. The leg, wing, and keel bones are important matrices for bone health which can be assessed using in vivo (palpitation, radiographs, virtual biomechanical analyses, blood assessments and computerized tomography (CT) scans of restrained birds) and ex vivo (using CT or micro – CT and image software, ashing and mineral analysis, cortical geometry) techniques (Campbell, 2021). The extent of bone health problems in laying hens has been reported, but the variation within a population of hens kept under common housing conditions has not been explored. In non-caged housing systems the hens have increased mobility which strengthens the bones (Dikmen et al., 2016) and this may have beneficial impact on tibia and keel bone integrity compared to conventional cages (Regmi et al., 2016). A higher tibia breaking strength was also reported in free-range system hens compared with both conventional cage and enriched cage systems (Dikmen et al., 2016). An increased level of damaged (and repaired) keel and fulcrum bones in non-cage systems have been reported by Gregory & Wilkins (1996), which can be linked to collision and landing

accidents during flights. However, the modern hen has been developed to have a smaller wing size to body ratio which increases wing loading compared to the jungle fowl (Moinard et al., 2004; Stratmann et al., 2016). Conventional caged layers on the other hand, have restricted space and movement which affects bone reabsorption and new bone formation, resulting in osteoporosis (Whitehead & Fleming, 2000; Lay et al., 2011; Campbell, 2021). However, they are shielded from keel-bone damage which is painful, affects hens' behaviour and reduces hen productivity and egg quality. Bone health impairment is a global layer welfare concern, with up to 95% of all birds regardless of housing system, having some degree of keel bone damage at the end of the laying cycle (Wilkins et al., 2011; Regmi et al., 2016; Saraiva et al., 2019). However, this can be improved by genetic selection and management strategies to incorporate opportunities for more exercise. More research is merited to explore the effects and control of various health damage in hens raised under various housing systems and also to develop more strategies to control bone injuries in commercial layer flocks.

1.1.2.2 – Incidences of pest, parasite, and disease load

An assessment from a sustainable development perspective has shown that conventional cage housing systems have the advantage of more disease-free hens compared to cage alternative systems (De Boer & Cornelissen, 2002). The studies from Appleby & Hughes (1991) also reported fewer cases of coccidiosis, while Abrahamsson & Ragnar (1995) observed less bird aggression and cannibalism in caged hens compared to non-caged hens. However, increased abdominal fat pad deposition and Fatty Liver Haemorrhagic Syndrome (FLHS), a metabolic disease that affects the liver has been reported to be associated with caged hens than non-caged housing systems (Shini & Bryden, 2009; Shini et al., 2019). The investigation by Jiang et al (2014) reported that providing perches in colony caged housing resulted in a reduced

abdominal fat pad weight, however, perches did not have an effect in reducing liver fat content nor the activities of serum alanine (ALT), aspartate transaminase (AST) which are enzymes that improves liver function. The study by Dikmen et al (2016) reported that differences in housing system did not affect blood glucose, total cholesterol, triglyceride, and calcium values. However, a worsened body wound score, higher mortality ratio but higher Heterophil/Lymphocyte ratio was seen in hens from caged housing compared to non-caged housing, while a worse footpad lesions but better feather score and higher blood phosphorus levels were observed in free range hens compared to conventional cage systems. In barn systems, there is a higher possibility of poor air quality which can affect the respiratory system of the hen (Guarino et al., 1999), a situation which is rarely observed in caged systems where faecal droppings go through wire mesh flooring and are collected and discarded automatically from the manure belt. With regards to bacterial contamination, the study of de Vylder et al (2009) indicated that cage housing systems did not pose a risk for *Salmonella* contamination, however, Gast and their colleagues, detected an 8-week faecal shedding of *Salmonella Enteritidis* post-inoculation, in enriched colony cages, compared to 10 weeks shedding observed in conventional cage housed hens (Gast et al., 2015). They however noted that this variation in bacteria shedding did not persist in the longer term. The study by Fossum et al (2009) reported that from 2001 to 2004, the occurrence of viral diseases was also higher in non-caged housing system and more hens from litter based free range housing system were submitted for necropsy and had a higher incidence of bacterial and parasitic diseases and cannibalism compared to caged birds. Poultry red mite, *Dermanyssus gallinae*, is a blood-feeding ectoparasite which adversely affects health and welfare, reduces egg production, and egg quality, increases aggressive feather pecking and causes anaemia and eventually death. It has the potential to affect hens in all housing systems but its occurrence is seen to be higher in free range /outdoor raised hens compared with either barn or cage systems (Guy et al.,

2004; Gunnarsson, 2017). The study of Höglund et al (1995), also reported about 4% of red mite affliction in cage systems, whereas 33% and 67% was reported in deep litter and backyard flocks respectively. Some common measure to control the incidences of red mites are the use of silica based acaricide preparations, cleaning of hen houses, the use of physical traps to collect larvae and insects, heat treatment of layer houses between consecutive laying cycles (Mul et al., 2020), inert dusts which targets and kills hosts by desiccation (Kilpinen & Steenberg, 2009). The study of Stafford et al (2006), had also reported that the use of intermittent lighting may be a promising measure to control red mite infestations in hen housing.

1.1.2.3 – Social interaction and natural behaviours

Hens exhibits natural behaviours such as pecking, wing flapping and dust bathing. Beak trimming, a widely used across the egg industry to prevent injurious pecking has been considered as a welfare concern as it contributes pain and sensory loss to the birds, and limits their ability to pick up food, build nests and interact with other birds. There has been an ongoing dialogue to put an end to de-beaking in egg production and four European countries (Sweden, Norway, Denmark, and Switzerland) have successfully passed legislation to end beak trimming. Although behaviours like gentle pecking is required for social recognition and interaction, the risk of extreme and harmful behaviours such as aggressive pecking (Campderrich et al., 2017), feather pecking (Huber-Eicher & Sebö, 2001; Sherwin et al., 2010; Brunberg et al., 2011), toe pecking (Leonard et al., 1995; Krause et al., 2011), vent/cloacal pecking (Pötzsch et al., 2001; Sherwin et al., 2010) may develop at a high frequency in non-cage systems and can worsen to cannibalism causing severe wounds, and death (Rodenburg et al., 2013). In conventional cage housing systems, the limited physical space reduces the ability of hens to perform their natural behaviours which can cause

inactivity, obesity, and physiological stress. Nonetheless, the study by Frizzell et al (2017) reported that environmental stressors was present in either conventional cage or furnished cages and it did not affect muscle colour, and muscle pH. Newberry et al (1988) documented that the use of increased light lux may have a beneficial effect on hen welfare by reducing bruising. Another study by Huber-Eicher et al (2013) recorded that red emitting diode lights reduced aggressiveness compared with white light in domestic Brown Nick hens. Including fibrous content in hen diets such as feather meal have been suggested to decrease aggressive feather pecking bouts (Harlander-Matauschek et al., 2006), and at 10% of the diet, feather meal improved plumage condition of the back area (Kriegseis et al., 2012). More research is still required to address aggressive social behaviours in hens and the review by Michel et al (2022) forecasts a future where the technology of sensors placed in individual hens will be deployed for timely and accurate detection of extreme behaviours in hens, in order to limit the spread of injury and death in flocks produced in non-cage housing systems.

1.1.2.4 – Hen performance and productivity

It has been well established that type of housing system considerably affects performance and production traits such as egg weight, feed efficiency, daily feed consumption and mortality (Taylor & Hurnik, 1996; Suto et al., 1997). However, the outcomes on studies to evaluate these differences have been inconsistent. Body weights were seen to be higher in conventional cages compared to furnished cages (Frizzell et al., 2017), while feed consumption, egg production, egg mass and dirty egg proportions were seen to be higher in free range systems and comparable in conventional and enriched caged systems (Dikmen et al., 2016). The study by Ahammed & Ohh (2013) recorded higher feed intake and egg weights in barn housed hens compared to conventional cages, however they observed that housing did not affect the daily egg production, body weight, egg mass and feed efficiency.

The outcomes of Englmaierová et al (2014) study showed that hens raised in conventional cages had higher egg production, feed intake and lower FCR compared to litter systems while Mostert et al (1995) reported higher day egg production, egg mass, better FCR, less mortalities and egg abnormalities than the free range and barn systems. In organic systems where hens have free access to the range, rearing intensity is lowered, egg production was reported to be lower and FCR was worse compared to conventional cage facility (Küçükyilmaz et al., 2012).

1.1.2.5 – Internal egg quality

The quality and safety of the egg are important measures of evaluation in the egg industry. Housing systems have been reported to affect egg quality and safety traits however the results from studies assessing these components have been inconclusive. Higher albumen height and Haugh unit were seen in eggs from caged hens compared to barn eggs, while shells were thicker in barn eggs than in cage eggs. The shape index, breaking strength, blood spots and yolk index were however not affected by housing systems (Ahammed & Ohh, 2013). Englmaierová et al (2014) also reported higher albumen qualities but higher eggshell weight in conventional cages, while eggs from enriched cages laid eggs with a higher yolk index. In organic systems, Küçükyilmaz et al., (2012) reported that albumen height, haugh unit, yolk ratio and shell weight were higher however, the yolk colour score was lower than that of conventional eggs. Although the findings from Zemková et al (2007) reported higher concentration levels of cholesterol in the yolks of eggs from litter raised hens compared to enriched cages, Küçükyilmaz et al (2012) reported that housing systems had no effect on egg yolk cholesterol concentration. Housing systems also affected total bacteria count on the egg surface and microbial contamination, with eggs from conventional and enriched cages,

having lower values for microbial contamination of *Enterococcus* and *Escherichia coli*, compared to barn eggs (Küçükyılmaz et al., 2012).

1.1.2.6 – Production economics

Profitability is a major criterion defining housing systems choices in egg production enterprises. The study by Matthews & Sumner (2015) reported that feed costs per dozen eggs, capital costs per dozen eggs, labour costs and total capital investments per hen-capacity were higher in the barn system, resulting in a 36% increase in the average total costs for egg production compared to conventional cage systems. Energy and miscellaneous costs formed a minimal part of total operating costs and was not affected by housing systems. In the study by De Boer & Cornelissen (2002), they reported that egg production under cage housing system are more economical compared to deep litter barn systems and the review by Michel et al (2022) documented that non-caged housing can lead to economic losses as a result of severe feather pecking, feather loss and increasing temperature loss, which can result in increased food consumption in defeathered birds. The difference in the cost of production leads to cheaper cost of eggs from caged housing and more expensive eggs from non- caged housing systems.

1.1.2.7 – Environmental effects

Egg production has effects on the environment, and potentially contributes 2.2 kg of CO₂ emission per dozen eggs, or 1.6 kg of CO₂ equivalent emission per kg of eggs (assuming average egg weight of 60 g) (Taylor et al., 2014). This contributes to acidification of forests and eutrophication of surface water. While individual studies on the emissions of ammonia, greenhouse gases and particulate matter from commercial laying hen houses have been carried out, there is limited information on the comparison of different housing systems on the environment. The study by Shepherd et al (2015) reported that house level methane

emissions were similar for all housing systems while house level ammonia and particulate matter emissions were highest in aviary systems compared to both enriched and conventional cage systems as a result of hen activities on the litter floor. In battery cages, faecal matter is collected on the manure belt and although ammonia emissions from the manure storage accounted for 60 to 70% of the farm-level emissions (Shepherd et al., 2015), in battery cages these can be more easily managed and converted to mechanical energy and used to power farm engines. More research is however needed to determine welfare-friendly, environmentally sustainable, albeit cost effective system of housing in commercial layer enterprises for improved hen performance, egg quality and egg safety.

1.2 Hen performance traits

1.2.1 – *Feed intake*

Feed is the major denominator in livestock enterprises. Its uniform consumption in flocks is important in optimizing cost and production efficiencies. On the other hand, overfeeding has been reported to cause erratic laying, increased incidence of double ovulations, oviduct involution of hens (Walzem et al., 1993), shell defects and follicular collapse. Several strategies have been explored to control over feeding in hens including quantitative measures such as reduced feed allowance offered multiple times a day (Taherkhani et al., 2010), skip-a-day feeding (Wilson et al., 2018), and time-restricted feeding (Saibaba et al., 2021), as well as qualitative methods where birds have access to different nutrient (protein, energy, or amino acids) allocations in diets (Ghazanfari et al., 2010) or feed dilution with low nutrient value ingredients (Rezaei & Hajati, 2009; Röhe et al., 2018). The feed intake of hens can be affected by various management and environmental factors. An increase in shed temperature decreases feed intake as hens are seen to stay off feed to avoid the creation of excessive body heat as a result of feed digestion and nutrient absorption (Mashaly et al., 2004; Franco-Jimenez et al., 2007; Song et al., 2012). Some studies carried out to investigate the effect of photostimulation on feed intake, showed that increasing light duration or intensity beyond the normal recommendation does not affect feed consumption (Newberry et al., 1988; Ma et al., 2013). Stocking density and housing systems is also an important factor however, the results from studies that investigated its effect on feed intake are varied. Whereas Carey, (1987) and Cook & Xin, (2005) reported that stocking density did not affect feed intake in caged hens, the studies of Mtileni et al (2007) and Kang et al (2016) found that feed intake was retarded with an increase in stocking density in floor pens. Geng et al (2020) observed that feed intake in Beijing You Chicken raised on floor pens were not affected by a stocking density of up to 7 hens/m² however, a stocking density of 8 hens/m² had an adverse effect on

the performance and welfare status of the birds. Mirfendereski & Jahanian (2015) concluded in their study, that a dietary supplementation of chromium – methionine complex can alleviate harmful responses to stressful conditions, improving feed intake and other laying performance traits. Maysa et al (2022) also reported that supplementing hen diets with 0.5% liquorice improved feed intake and other hen performance traits among floor pen hens with a stocking density of up to 9 birds /m². The feed consumption in hens can also be affected by physiological factors including body weight (Harms et al., 1982; Leeson & Summers, 1987; Lacin et al., 2008; Pérez-Bonilla, et al., 2012), where heavier hens are seen to consume more amount of feed, and disease conditions, where immune compromised hens are seen to consume less feed than healthy birds (Hagger et al., 1989). Temperature, feather cover and housing system can affect the nutrient requirements of laying hens. Differences in protein and energy levels in the diet can also influence the rate of feed consumption. Whereas increased dietary energy level led to a decrease in feed intake (Gleaves et al., 1973; Ivy & Gleaves, 1976; Grobas et al., 1999b; Panda et al., 2012; Clark et al., 2019), differences in dietary protein levels did not seem to have an effect on feed intake in hens (Gleaves et al., 1973; Salama & El-Sheikh, 2012).

1.2.2 – Body weight

Hen body weight is an indicator of feed consumption and nutrient utilization and is important for sexual maturity (Chen et al., 2006), skeletal development, and egg production (dos Santos et al., 2017). Thus, the hen requires efficient deposition of protein, fat and other nutrients in the right amount for each growth stage. The growth of the hen for the first 18 weeks is usually divided into three phases: 0-6 weeks characterised by bone and muscle formation; 6 to 12 weeks for feathering and the reproductive tract development phase which takes place between 12 to 18 weeks of age (Neme et al., 2006; dos Santos et al., 2017). The expected growth rate curve of ISA Brown hens from 0 – 90 weeks is presented in Figure 1.5. Hens begin

exponential weight gain from day old and attain mature body weight of about 1700 1880 g $\pm$ 10% at about 40 - 41 weeks of age, which they usually maintain until late laying stage. The study of Robinson & Robinson (1991) submitted that differences in body weight attained at an early stage was maintained until 62 weeks of age, with the high-weight hens at early lay being heavier than the low and medium weight hens at the end of the study.

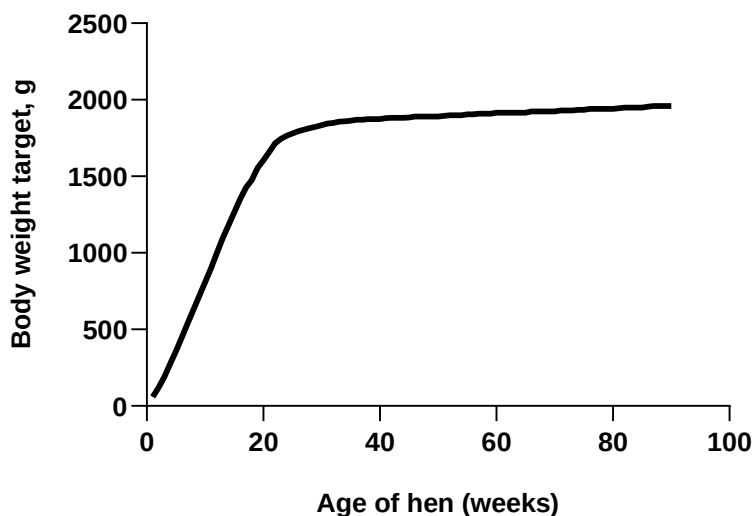


Figure 1.5 - The target growth rate curves of ISA Brown hens from day old to 90 weeks of age. Source, ISA Brown, (2017).

Although hen body weight plays a significant role in reaching sexual maturity and egg production it is not as important after the climax of the reproductive phase has been reached. Thus, birds with a lower body weight which come into lay relatively fast may be preferable as they have a lower feed requirement thus lower maintenance rate and also a shorter non-productive phase prior to sexual maturity (Grossman & Bohren, 1985; Di Masso, Dottavio, Canet, & Font, 1998). There is a positive genetic correlation between body weight and egg weight (Siegel, 1961; Festing & Nordskog, 1967), however, it may be more economical to have light weight early maturing hen which lay consistently (Poggenpoel & Duckitt, 1988), compared to heavy hens which lay almost the same size or sometimes extra-large eggs, which

are not always preferred by consumers. Therefore, controlling hen body weight while maintaining commercially acceptable egg weights is necessary to maintain uniformity and optimise production resources (Nordskog & Briggs, 1968). Excessive body weight as a result of overfeeding has been flagged as the major cause of fatty liver haemorrhagic syndrome (FLHS). This syndrome can be characterised by excess infiltration of fat in the liver, enlarged combs and wattles with dandruff-like appearance (Harms et al., 1972) and obvious liver haemorrhages upon necropsy (Couch, 1956; Harms et al., 1972; Grimes et al., 1991; Shini & Bryden, 2009b). The results from Jiang et al (2013) indicated that a high energy-low protein diet induced a fatty liver disorder in laying hens. The study of Walzem et al (1993) reported that overfed hens had a 33% incidence of FLHS, and this was indicated by the presence of severe liver haemorrhage score, and definitive lesions following a hepatic reticulin. Trott et al (2013) also reported that 97% of FLHS affected birds were found to have large fat depots or were obese. Body weight in hens also influences the deposition of abdominal fat and influences the size of digestive organs. The study of Chen et al (2006) reported that body weight, liver weight, and abdominal fat weight were higher in over-fed hens while Dastar et al (2016) recorded heavier ovaries compared to lighter weighing hens. Fat is a nutritionally expensive tissue (Jiang et al., 2014; dos Santos et al., 2017) and so effort has to be made to ensure that excessive deposition of fat above the level that is physiologically required is avoided as this can influence hen performance, productivity and health.

1.2.3 – Egg production

Egg production in hens is an important measure of productivity in the layer industry. It is calculated as the percentage number of eggs laid per particular period, and it's heritability up to 275 d old was estimated to be 14% (Poggenpoel & Erasmus, 1978). Various factors may influence egg production in laying flocks including age of hen, breed, body weight of hens, nutrition as well as housing, environmental and disease conditions. Tůmová et al (2017)

reported that the number of eggs laid by a hen during her reproductive life are dependent on the changes that occur in the internal cycle length over time. Egg production decreases due to the increase in degeneration of ovarian follicles which occurs during ageing (Waddington et al., 1985; Bahr & Palmer, 1989). The findings from Barua & Yoshimura (2001) revealed that in late laying hens, ovarian tissues antibodies begin to appear in the blood circulation and an increase in the concentration of these autoantibodies is inversely associated to egg production. According to the ISA Brown breed expectation (ISA Brown, 2017), by 17 weeks of age, it can be expected to start seeing some eggs produced. However, the production rate is usually <10% at this age but increases to up to 85% by 22 weeks. Peak production of >90% is estimated to be reached at 23 weeks and hens usually maintain this rate until about 50 weeks of age, when it begins to gradually decline to <90%. Jacob et al (2011) also records that production gradually declines to about 65% after 12 months of lay and that the total egg production of a flock of hens at a particular age is determined by the individual patterns of sequential laying at that time (Johnston & Gous, 2003). The study by Hocking et al (2003) to determine the extent of variation on traditional and commercial lines revealed that there were no detectable differences for rate of lay between the two categories. Body weight uniformity at sexual maturity and during laying life plays an important role in egg production (Leeson et al., 1997). The study by Harms et al (1982) reported no difference in egg production based on hen body weight, however a more recent study by Lacin et al (2008) reported that egg production rates were higher in light weighing Lohmann White hens compared to the heavy group. In the second cycle production of moulted ISA Brown hens ranked as light, mid-light, mid-heavy and heavy weight hens, Ocak et al (2004) reported that there was no differences in the rate of egg yields in the groups except the heavy group which had the least egg yield. Mashaly et al (2004) reported that heat stress caused by high temperature and humidity significantly inhibited egg production and also affected the

immune function of commercial hens. Nutrition also plays an important role in egg production. Grobas et al (1999a) reported that an increase in supplemental fat from 0 to 4% improved egg production however an increase in the linoleic acid content of the diet beyond 1.15% did not affect egg production, while Pérez-Bonilla et al (2012) noted that an increase in the concentration of the apparent metabolizable energy in the diet of Hyline Brown hen up to 2,850 kcal/kg, increased egg production. The study by Jaroni et al (1999), reported that egg production was not affected when enzymes - xylanase and protease were added to a high fibre diet in late laying birds.

1.2.4 – Age of first egg.

The age of first egg (AFE) is an important indicator of sexual maturity in hens and it is associated with reproductive and production performance. The AFE may be affected by several factors including breed, body weight, photo stimulation and environmental seasons, feed intake and disease conditions. Exposure to light has been documented as a key determinant of AFE in hens, with delayed onset of laying seen in the winter months (shorter daylight) compared to summer season (longer daylight). The study by Lewis & Gous (2004) reported that hybrid hens requires more than two cycles of long days to initiate rapid gonadal development and that the mean AFE of hens was earlier by 2 to 3 weeks following an increased photo period from 8 to 14 h. An earlier study by Lewis et al (1997) also documented that increasing photoperiod from an 8 h length was effective in advancing AFE when given at 63 or 84 days of age. This was in agreement with the findings from Lewis et al (2001) who documented that ISA Brown pullets photostimulated for 16 h at 44 and 54 days of age, matured at least 3 weeks earlier than hens on a short-day photostimulation of 8 h. They however noted that an injection of oestradiol between 28 to 40 days of age was seen to delay the AFE of birds photostimulated at 54 d. The presence or development of persistent infection in pullets can affect the sexual maturation and AFE. The study by Wigley et al (2005) showed

that there is an association between the loss of T-cell activity and the onset of egg production, where a non-specific suppression of cellular responses caused by *Salmonella pullorum* and *Salmonella enteritidis*, can lead to reproductive tract infection, delayed laying and transmission of the bacteria to the egg causing table egg contamination and potential concerns for public health. In a study crossing local and exotic hens, AFE inheritance was seen to be influenced by the sire of the cross bred group (Udeh, 2010). Breed like Leghorns, Barred, Delawares, Rhode Island Reds, ISA Browns and Australorps which are usually smaller in weight, lay sooner at about 18 to 20 weeks of age, while heavier breeds like Plymouth Rocks, Orpingtons, and Wyandottes tend to lay later at about 28 weeks of age. The Golden Comets is reported to start laying as early as 16 weeks. Feed intake has also been reported to be a driver of AFE and hens fed every day (Hurwitz & Plavnik, 1989; De Beer & Coon, 2005), or with a high energy diet (Pearson & Herron, 1982), have been reported to reach sexual maturity at a younger age than other groups. Moderate time-restricted feeding strategy of up to 8 h have been reported to delay onset of lay by 4 days compared with ad libitum feeding. Hens on ad libitum feeding were reported to reach sexual maturity with 11.0 large yellow follicles compared to 7.1 follicles in restricted fed birds (Renema et al., 1999). AFE was reported to be delayed in hens previously kept on a skip two days feeding regime (Bartov et al., 1988), and in hens restricted for the first 24 weeks and transitioned to *ad libitum* thereafter, AFE was delayed by up to 17 days (Robbins et al., 1986). Body weight plays a critical role in influencing the age of first egg lay in hens as a certain body weight threshold is needed for pullets to reach follicular maturation and egg production. Body weight and/ or abdominal fatness was reported as a potential determinants of the onset of egg production in broiler breeder hens (Bornstein et al., 1984) and the study by Renema et al (1999) reported that heavier birds enter into lay 7 days earlier than lighter weighing hens. This is similar to the findings from Robinson & Robinson (1991) who documented that low-weight broiler

breeder pullets came into lay later than the medium and high weighing pullets. Ciacciariello & Gous (2005) also agreed with the findings and reported that heavy weight breeder hens exhibited a significantly advanced sexual maturity. From past studies, it can be agreed that feed intake and body weight are important to stimulate egg production, however, hens should not be over fed to begin laying sooner as this may affect total egg numbers, peak rate of lay, mean egg weight, trigger reproductive problems for the hens and can lead to the production of abnormal eggs of poor internal quality (Ciacciariello & Gous, 2005).

1.2.5 – Weight of egg at first lay.

Today's hens have been modified through selective breeding to come into lay earlier, and there can be consequences of this improvement on the weight of the first egg, weight of early eggs and even on the egg weight throughout the laying life. The early studies of Hadley & Cladwell (1920) found a tendency for the birds whose first egg had a higher weight to produce eggs whose mean annual weight was also proportionately higher, while Atwood (1923) reported a positive correlation between mean weight of the first ten eggs laid and mean annual egg weight. Extensive study hasn't been carried out to understand the causal factors and effects of varying weights of first egg, however, age of maturity, hen body weight, genetics, photo stimulation, feed intake and nutrient content in the diet have been identified as some factors which influences the weight of first egg. The study by Summers & Leeson (1983) reported that early maturing hens produced a higher percentage of small eggs and heavier pullets produced first eggs with lower egg weight. Bornstein et al (1984) also reported negative correlations between age at first egg and body weight, abdominal and skin fat concentration. The study by Bruggeman et al (1999) showed that birds feed restricted from 7 to 15 weeks of age had higher proportional weights of ovary and oviduct at age of sexual maturity than birds on an *ad libitum* diet. Summers & Leeson (1983) noted that slight changes in the protein, methionine and linoleic acid content of the diet did not affect the size of early

eggs, and Ciacchiariello & Gous (2005) reported that photostimulation did not have a positive effect on the weight of initial egg in broiler breeders as hens on an 8h constant photoperiod had the heaviest first egg compared to hens exposed to up to 16 h photoperiod at 19 weeks. A dated study by Godfrey (1933) summarised that mean annual egg weight can be determined at the beginning of the pullet laying year from a knowledge of the average weight of the first ten eggs. Therefore, although early maturing pullets are attractive to producers from an economic and inheritance standpoint, it is important to examine the effect of early maturity on early egg size, egg size in the laying life, persistency in lay and also on the health and welfare of the hen.

1.2.6 – Egg weight

Egg weight is an important parameter of choice not only for consumers, but for egg producers as well. It is an crucial gauge of productivity for producers - influencing sorting, grading and price, preference for consumers influencing choice and nutritional content and performance in the hatchery - influencing weight of day old chicks (Khan et al., 2004). Egg weight is influenced by age, breed, housing conditions, disease conditions, body weight, nutrition, and dietary composition. The age of the hen is a major factor affecting weight of egg with older birds laying heavier eggs (Silversides & Scott, 2001; Rizzi & Chiericato, 2005; Rizzi & Cassandro, 2009; Akyurek & Okur, 2009; Škrbić et al., 2011 and Tůmová et al., 2017), however, Chung & Lee (2014) reported that there was no difference in the weight of fresh eggs laid by 40 and 60 week old commercial hens. The increase in egg weight with increase in hen age is a result of the increase in components of the egg (Mohamed Ketta & Tůmová, 2018). Breed and housing conditions are also influencers of egg weight. Past studies have reported no significant difference in egg weights from ISA Brown hens (Rizzi et al., 2006; Zemková et al., 2007), HyLine Brown (Anderson, 2010; Wężyk et al., 2012), Ancona (Mugnai et al., 2009) and Lohmann (Küçükyilmaz et al., 2012) which were raised on different

rearing systems. However, the study by Küçükyilmaz et al (2012) reported that white – shelled layers laid heavier eggs compared to the brown layers in both organic and conventional production systems. Hanusová et al (2015) also reported that Oravka breed eggs were heavier than eggs of Rhode Island Red breed. The nutrient composition of the diet might influence the size of eggs produced. The study by Ebtsam et al (2017) showed that including carotenoid enriched *Spirulina* algae in hen diets increased egg weights regardless of hen strain. Zhang et al (1991) also found that feeding raw, conventional, full-fat soybeans and raw Kunitz trypsin inhibitor-free full-fat soybeans increased egg weight in commercial layers aged 35 weeks. Pérez-Bonilla et al (2012) also reported that hens whose dietary energy concentration were increased were heavier and produced bigger eggs compared to lighter hens. The study from Najib & Al-Yousif (2014) reported that inclusion of corn oil (linoleic acid) up to 4% in the diet without jeopardizing the energy content of the ration increased egg size of Saudi local brown birds during the onset and first part of the laying year. Some other factors which may influence egg weight includes environmental conditions such as temperature (Talukder et al., 2010), the position of an egg in a laying sequence and the oviposition time. The study of Patterson (1997) showed that oviposition time influenced egg weight where eggs laid in the morning were consistently heavier by 2 - 9 g per egg per day, than those laid throughout the remainder of the day.

1.2.7 – Feed conversion ratio

Feed to egg conversion ratio (FCR) is defined as the amount of feed consumed by the hen to produce a unit mass of egg. It is an important trait in commercial layer production, widely used for breeding and selection purposes (Tolkamp et al. 2010; Tu et al. 2011), where lower ratios of FCR indicates a higher nutrient and energy efficiency. Differences in feed efficiency can be linked to variations in hen productivity and performance, and a bird is considered an efficient producer when it is able to use a relatively larger part of feed intake for egg

production. Due to progressive genetic and nutrition interventions, the feed efficiency of hens has advanced tremendously over the years, positively impacting hen performance, productivity, hen health and welfare as well as the environment (Hume et al., 2011). Nonetheless variation in the feed efficiency of hen flocks are still present and a life cycle analysis study on the U.S. egg industry, conducted by the Egg Industry Centre, reported an FCR range of 1.76 to 2.32 among birds reared for eggs in the United States (Pelletier et al., 2014). Feed efficiency is a moderately heritable trait ($h^2 = 47\%$) that is more dependent on feed intake rather than egg mass (Wolc et al., 2013). The FCR of a hen can be affected by various factors such as genetics (Mugnai, 2011; Hume et al., 2011; Wolc et al., 2013), heat stress (Song et al., 2012; Attia et al., 2016) plumage or feather cover (Hagger et al., 1989; Su et al., 2006) and body weight of the hen (Harms et al., 1982; Ocak et al., 2004; Lacin et al., 2008; Gordon et al., 2009; Pérez-Bonilla et al., 2012; Pérez-Bonilla et al., 2012). The work from Leeson & Summers (1997) reported on the relationship between feed efficiency and carcass composition, ability to metabolize dietary energy, bird activity, and feather cover and they found that hens classified as efficient were smaller, consumed less feed per gram of egg mass, were lighter in weight than intermediate and inefficient hens and had a better plumage. The study by Lewis & Gous (2006) also concluded that breeder hens which were poorer feed to egg converters at 20 weeks had a heavier body weight and laid more 'unsettable' eggs compared to the more efficient hens.

1.3 Egg quality traits

Egg quality can be defined as the characteristics which ensures safety, nutrition and contributes towards better economic value of the egg (Stadelman, 1977). It is important for nutrient preservation, prevention of microbial contamination and embryo survival (Du et al., 2015). The quality of the egg can be determined externally via the weight of the egg, shape index, and shell characteristics and internally through albumen and yolk parameters. In addition, the absence of external and internal abnormalities are also measures of quality of the egg. External egg quality assessments are conventionally done on intact eggs, however, internal assessments are typically done on broken out eggs while egg breaking strength measurements leads to the destruction of whole eggs. The review of Narushin (1997) and the study of Alaşahan & Günlü (2012) reports on assessing internal egg quality measurements on intact eggs and they concluded that non-destructive methods are advantageous as it reduces loss. In addition, it has potentials of reduced error rates and so correct estimates during production can be easily made. Egg quality can be influenced by several factors including bird strain (Silversides & Scott, 2001; Monira et al., 2003; Vits et al., 2005; Joubrane et al., 2019), age of hen (Akyurek & Okur, 2009; Samiullah et al., 2014), nutrition (Bar et al., 2002; Jamroz et al., 2003; Saminathan et al., 2020), housing system (Tumová & Ebeid, 2005; Dukic-Stojcic et al., 2009; Samiullah et al., 2014; Mohamed Ketta & Tumová, 2018; Küçükyilmaz et al., 2012), egg storage time, temperature, and relative humidity (Sills, 1974; Sauveur, 1976; Samli et al., 2005; Oliveira et al., 2009; Akter et al., 2018; Feddern et al., 2017), contamination of the egg including from mishandling, improper packaging, egg contact with contaminated surfaces (De Reu et al., 2006a; Stringhini et al., 2009; Mendes et al., 2012), oviposition time (Tumová & Ebeid, 2005; Samiullah et al., 2016) and disease status of the hen (Williams, 1992).

1.3.1 – External egg quality measurements

Asides from being an indicator of hen performance, the weight of the egg is an important measure of egg quality and profitability for producers, and a yardstick for preference for consumers. In the US, eggs are graded based on their weights into jumbo (≥ 70 g), extra-large (65 – 70 g), large (56 – 65 g) and medium (49 – 56 g). In Canada, this grading system is similar with a slight modification thus: small (42 – 48.9 g), medium (49– 55.9 g), large (56 – 63.9 g), extra-large (64 – 69.9 g) and jumbo (70 g or higher) (USDA, 2000; FAO, 2003). The height or length of the egg is the vertical distance between the top of the pointed end of the egg and the tip of the bottom end of the egg. It is the measured in millimetre using graduated vernier callipers. The width of the egg is also measured in millimetre using a graduated vernier calliper as the distance around the largest diameter of the egg. The shape of the hen egg is an important measure of quality and of significant interest to the layer industry. The ideal egg shape is oval with one end pointy and the other end round. It has been described using the shape index which is expressed in percentages and calculated as shape index = $W/L \times 100$, where W is the width, mm and L is the length, mm of the egg (Nedomova et al., 2009). The shape index of the egg has been standardised and is classified into sharp, normal/ standard or round. Sharp eggs are characterised by a shape index of $<72\%$, normal or standard, is when the shape index is between 72% and 76%, and round is when the shape index is above 76% (Duman et al., 2016). Sharp or round eggs, regardless of the weight or size, are not graded as AA or A due to their odd appearance, inability to fit in standard egg cartons, and the high tendency to get broken or damaged during shipment compared to normal or standard shaped eggs. The study of Shi et al (2009), showed that egg shape index is significantly and positively associated with egg weight. Shape index has a significant effect on the proportion of shell breaking strength variation and affects the risk of cracked eggs (Anderson et al., 2004). The specific gravity of the egg is a technique which follows the saline

flotation method of Hurnik et al (1977), to determine egg freshness. To measure the specific gravity of egg, saline solution with different concentrations of sodium chloride (NaCl) are made and the concentrations are measured with a hydrometer. The eggs to be assessed are placed in the saline solutions and eggs that sink in solutions irrespective of the saline concentration, still have smaller air sacs and thus indicates freshness and higher egg quality. Eggs with a larger air sac, sinks in saline solutions and are considered stale.

1.3.2 – Albumen parameters

Albumen quality is a leading economic trait in the egg industry which can be evaluated by several parameters including the amount of albumen (Holst & Almquist, 1931), albumen width, albumen height (Wilgus & VanWagenen, 1936), Haugh unit score (Haugh, 1937), the albumen index (Heiman & Carver, 1936) and the albumen pH. The albumen contributes about 58 – 64% of the total egg weight and is directly proportional to the weight of the egg, with bigger eggs having a higher amount of albumen. The albumen which contains approximately 88% water, 11% proteins and 0.2% cholesterol is made up of the inner thick and outer thin layers. Ovalbumin, ovotransferrin, ovomucoid, ovomucin, lysozyme, and globulins, make up approximately 90% of the total albumen protein. These proteins are involved with various biological functions including antimicrobial activity, structural and gelling characteristics, immune responses, protease inhibition, and metal ion binding or transport, which may play an important role in influencing albumen quality. Ovomucin is the most important egg albumen protein. It makes up 1.5 to 3.5% of the total albumen protein and is responsible for the thickness and structure of the inner thick egg albumen which plays a critical role in preventing bacterial movement within the egg white (Toussant & Latshaw, 1999; Strixner & Kulozik, 2011). A thick and generous amount of albumen maintains the egg yolk in the centre protecting it from physical shock damage and providing more protection against bacterial invasion to the yolk (Van Tijen, 1972). As the egg ages due to storage, the

inner thick layer of the albumen breaks down and moisture content of the albumen is lost to the environment through the pores in the shell or to the yolk through the yolk membrane, thus reducing its volume and its protective abilities. The albumen height of an egg is measured by the thickness or compactness of the inner thick albumen, expressed in millimetres. The thicker the albumen, the better the quality of the egg. The albumen height has a heritability of 48% (Poggenpoel, 1986) and is highest when an egg is freshly laid, decreasing with the age of hen, age of egg and storage time and conditions. Eggs with albumen heights of 8 to 10 mm are being considered of superior interior quality (Zeidler, 2002). The width of the albumen is also important for assessing quality. A higher quality albumen will have a smaller width, signifying a well-structured peptide bond of the proteins contained in the albumen. The majority of the study on albumen height (thickness) focuses on the thick albumen. However, the thin albumen is also a measure of quality with up to 5% of eggs from commercial flocks having an extremely thin, thin albumen. The study of Leeson & Caston (1997) reported that breeder hens fed a higher level of protein in their diet, laid eggs with a more compact thin albumen. They also observed that the offspring produced from the spreading thin albumen eggs has a higher shell thickness and so they proposed that the excessively large spreading thin albumen area is caused by eggs spending more time in the shell gland. Albumen height can also be corrected for egg weight in grams to determine the Haugh unit (Haugh, 1937). The Haugh unit, is a generally accepted measure of quality of the egg albumen and is the logarithmic function of the height of thick albumen adjusted for EW. The Haugh unit is calculated using the equation $HU = 100 \times \log_{10} (H - 1.7 \times W^{0.37} + 7.6)$, where H is the albumen height, mm and W is the egg weight, g. However, Keener et al (2006) reported that the electronic Haugh, which electronically measures albumen height and calculates HU, is a more accurate tool for measuring Haugh unit as it gave the highest quality measurement with the lowest coefficient of variation and was sensitive to quality loss at 5°C.

A higher HU value indicates a higher egg quality and according to the Quality Control Program of the United States Department of Agriculture (USDA), eggs may be classified as AA grade when they exhibit a HU value of 72 or higher. Eggs with a HU value between 60 and 72 are graded as A, while those with a HU value lower than 60 are considered of lower quality and graded as B (USDA, 2000). Although HU is generally accepted in the industry as a measure of albumen quality, its soundness has been questioned as a suitable technique (Eisen et al., 1962) and other researchers have suggested albumen height to be used as an alternative measure (Silversides et al., 1993; Silversides & Villeneuve, 1994). Albumen index calculated as the percentage ratio of the albumen height and width is also an important measure of quality. A higher albumen index suggests a higher albumen height and smaller albumen width, and this is an indication of the roundness of albumen around the yolk. As the egg ages, the albumen width becomes wider, and height flattens. This causes the coverage round the yolk to decline, and this can predispose the yolk to bacterial contamination. The pH of the egg albumen is also an important measure of quality, especially during storage (Silversides & Villeneuve, 1994). The albumen pH of a freshly laid egg is estimated to be between 7.6 to 8.5 (Heath, 1975; Heath, 1977). During storage, egg albumen releases CO₂ originating from carbonic acid dissociation, forming a buffer system within the albumen. The process is followed by alkalization, demonstrated by an increase in alkalinity up to 9.18 which causes changes in the structure of albumen (Stadelman & Cotterill, 1995).

Some major factors influencing albumen quality are age of the hen, housing conditions, storage time and conditions, egg weight, body weight and genotype. The albumen of an egg is at its highest quality in freshly laid eggs of young hens. Studies have confirmed that older hens lay eggs with a reduced albumen height and Haugh unit compared to younger hens (Perić et al., 2017; Marzec et al., 2019). The weight of the albumen increases with an increase in age (Rossi & Pompei, 1995; Suk & Park, 2001), however the percentage of albumen

proportional to total egg weight decreases as hens age (Van Den Brand et al., 2004; Rizzi & Chiericato, 2005). The study of Jin et al (2011) reported that increase in storage temperature and time decreased albumen weight and Haugh unit (HU) and increased albumen alkalinity (Marzec et al., 2019). However, refrigeration have been shown to preserve albumen quality parameters compared to exposure to room temperatures (María Elena et al., 2006). Also, oiling (Eke et al., 2013), ozone treatment (Choi & Lee, 2012), egg washing with disinfectants and antibacterial chemicals (Goto et al., 1996) as well as plasma activated water treatments (Lin et al., 2019) have been reported to slow down loss of albumen quality and provide protection to the eggs against bacterial invasion and degradation especially in tropical countries. Hen genotype has also been reported to influence albumen quality where eggs from ISA-Brown hens had a higher albumen pH and albumen height compared to those from ISA-White hens (Scott & Silversides, 2000). The effect of housing on albumen quality has been inconsistent. Whereas Englmaierová & Tumorová (2009) reported that eggs from litter system had a higher Haugh units, albumen index, albumen share, and albumen pH compared to eggs from caged hens, the studies of Petek et al (2009), Tumorová et al (2011), Ahammed & Ohh, (2013), Englmaierová et al (2014) and Jones et al (2014) have reported that caged hens laid eggs with a higher albumen quality compared to non-caged housing. Not much has been done to investigate the influence of body weight on albumen quality, however, the study of Lacin et al (2008) reported that body weight of hen influenced albumen index and Haugh unit parameters, where lighter weight hens lay eggs with a higher albumen quality. Pérez-Bonilla et al (2012) also reported that eggs from heavier Hy-Line Brown hens had a lower proportion of albumen than eggs from the lighter cohort and the Haugh unit has been reported to be highest in small, intermediate in large and lowest in medium size eggs (Shi et al 2009).

1.3.3 - Yolk Parameters

The yolk is the powerhouse of the egg, contributing up to 31% of the total egg weight, containing 48% water, 17.5% proteins and 32.5% cholesterol (Anton, 2007). The quality of the yolk is typically measured by the height, weight, colour, index, and the strength of the yolk membrane. The weight of the yolk is an important measure of quality. Bigger yolks are more appealing to the consumer and also for breeders as they have a higher hatchability rate (Nangsuay et al., 2011). The yolk height and yolk width are both important measures of quality with a higher value of yolk height and smaller value of yolk width indicating higher yolk quality. Yolk index is a measure of the spherical nature of yolk and determined by dividing the height of the yolk by its width (Stadelman, 1986). It was developed by Funk (1948), but refined by Kelly (2003) where measurements are done without the yolk being separated from the albumen. Physically, yolks from fresh eggs have a curved shape when put on a flat surface, but this flattens out as the egg ages (Kelly, 2003). A higher yolk index therefore implies an increase in quality (Fayeye et al., 2005), and just like most egg quality parameters, yolk index decreases with age of hen (Marzec et al., 2019). The yolk pH in freshly laid eggs is generally about 6.0, but with extended storage, and increase in storage temperature the alkalinity increases to between 6.4 and 6.9 (Stadelman & Cotterill, 1995; Samli et al., 2005; Tânia et al., 2010). A more alkaline yolk pH will negatively influence the vitelline membrane and albumen alkaline ions could be exchanged with yolk H^+ ions, leading to protein denaturation of the albumen and a weaker antimicrobial defence of the egg as a whole (Tânia et al., 2010). Yolk colour is an important visual measure of egg quality. It is usually decided using a yolk colour fan graduated from 1 to 15 or 1 to 16 depending on the brand of fan. The yolk colour pigments are determined by fat soluble xanthophyll which are typically present as lutein and zeaxanthin in diets and supplementary carotenoids (Lee, Kim, & Lee, 2001; Ituntaş & Aydin, 2014). Xanthophyll and carotenoids are absorbed in the

intestine together with lipids, therefore, based on the level of inclusion in the diet the colour of the yolk can vary from a very pale yellow to deep orange (Silva et al., 2005). Yolks with a darker colour are known to contain more fat-soluble deposits and thus are richer in fatty acids.

Certain factors have been associated with the quality of the yolk including rate of feed intake, dietary content, storage time and conditions, age of the hen, housing conditions, egg weight, hen body weight. The study of Suk & Park (2001) and Nangsuay et al (2011) reported an association between yolk weight and hen age, with older hens laying eggs with bigger yolks. The study of Perić et al (2017) have shown that as hen ages, the vitelline membrane weakens and water from the albumen penetrates the egg yolk to lighten the yolk pigments. Storage of the egg in high temperatures also affects the yolk colour of the eggs. Eggs from the heavier hens had a higher proportion of yolk than eggs from the lighter hens (Pérez-Bonilla et al., 2012). Ahn et al (1999) also reported that the percentage of yolks was increased with the inclusion of dietary conjugated linoleic acid. Housing also affects yolk quality; however, the results have been varied. Whereas higher yolk quality and cholesterol concentration was obtained in the egg yolk of hens raised on litter system compared to cage system (Zita et al., 2018), Jones et al (2014) reported no housing effects on yolk quality measurements, while Petek et al (2009) reported darker yolk colour and thicker eggshells for free range hens compared to conventional caged hens. Bigger eggs were seen to have a positive correlation with yolk height, yolk index and yolk colour (Şekeroğlu & Altuntaş, 2009).

1.3.4 – Shell parameters

The eggshell plays an important role of protecting the eggs from physical destruction, and pathogenic invasion from the environment (Hunton, 2005), as well as influencing consumer preferences and egg prices. Various researchers, (Alkan et al., 2008; Bhattacharya et al.,

2011; Ketta & Tůmová, 2016) have shown that the quality of the avian eggshell can be evaluated by its weight, thickness, breaking strength and colour. The shell makes up about 11% of the egg weight and is positively associated with the weight of the egg (Mohamed Ketta & Tůmová, 2018). The thickness of the shell is measured at the equator, in the middle of the egg using a digital micrometre. Measurements is usually taken after the shell membranes have been removed. The eggshell breaking strength, measured in Newtons or grams is the amount of force that needs to be applied to the shell of an intact egg until it breaks. The factors influencing the quality of the egg shell has been excellently reviewed (Roberts, 2004). Briefly, eggshell quality may be affected by proprietary products in the diet including zinc and manganese which act as cofactors or activators for enzymes that are involved in eggshell formation. It may also be influenced by production systems and flock densities, disease conditions of flock such as infectious bronchitis which causes pale coloured eggshells, stress including disruption during laying and heat stress which limits the availability of blood calcium for eggshell formation, nutrition such as calcium, phosphorus and vitamin D content of the diet, moulting, hen age and the hen genotype. Increasing feed particle size might improve hen performance however the results on effect of particle size on hen performance and egg quality is varied. Although the study of Garcia et al (2020) reported that the use of corn with particle size of mean geometric diameter of 1085 μm in rations does not affect the performance and eggs quality of Hisex Brown laying hens, the study of Ege et al (2019) reported that hens in enriched cages, hens fed a coarsely ground diet had a greater feed conversion ratio, higher yolk colour score, bigger livers, gizzards, pancreases and small intestine weights compared to hens a finely ground diet. On the use of limestone as a calcium source, the study of Świątkiewicz et al (2015) reported that the use of large particles increased eggshell percentage, thickness, density and breaking strength in 69 week old hens compared to fine particles of limestone. Similarly results were also found in the work of Guinotte &

Nys (1991) and Guo & Kim, (2012) who found that the egg weight in hens fed small particle size of limestone treatment were lighter, egg specific gravity was lower, and eggshell strength and eggshell thickness were lower when compared with other treatments that had larger particle sizes. Tůmová et al (2009) reported that eggshell strength was higher in hens raised in cages. The studies by Begli et al (2010) and Ketta & Tůmová (2018) reported that egg weight is positively associated with egg shell thickness and heavier eggs tend to have thicker and stronger shells. However on the contrary, Şekeroğlu & Altuntaş (2009) documented a negative association between egg weight and shell thickness because as the hens becomes older, the eggs become bigger and the spread of the calcium deposit becomes thinner thus decreasing it's thickness and strength. Shell colour is largely influenced by the breed of the hen, with hens with white ear lobes and white feathers, typically laying white-shelled eggs, while hens with brown ear lobes and coloured feathered laying brown-shelled eggs. The colour and brightness of the eggshell is measured using a reflectance colorimeter and studies have shown that brown shelled eggs have a bigger weight than white shelled eggs (Vits et al., 2005; Joubrane et al., 2019).

1.3.5 – Egg abnormalities

Abnormalities in the egg can be either external (on the shell) or internal (within the egg). Internally, egg abnormalities can be seen as displacements of the yolks from the central position or yolks stuck to the membranes, abnormal shaped or broken yolks, very thin albumen, presence of meat and blood spots, extra-large or running air cells, all which can be detected in an intact egg through careful candling (FAO, 2003). External egg abnormalities are seen mostly on the shell. As graphically described and represented by Alltech (2018) and summarised in Table 1.3, these abnormalities can be classified based on colour, stains, shell defects, texture of the shell, deposits on the shell and odd shape of the egg.

Table 1.3 - Description of some common external egg abnormalities

Category	Types of defects	Description and causes of defects
Colour	Pale coloured, lilac/pink coloured, brown speckled, white speckled	Pale coloured eggs are typical in brown shelled eggs and caused as a result of poor pigmentation of the cuticle deposited in the eggs. Pink eggs are due to an association between cuticle deposition and an additional calcium layer on the egg. These defects can be triggered by extra calcium in the diet, stress, and disease conditions.
Dirty	Excreta stained; blood stained	Eggs which are laid with smears or deposits of excreta or blood on parts or all of the egg. Excreta -stained eggs may be caused by wet droppings and diarrhoea, high indigestible feed ingredient while blood-stained eggs may be as a result of hens being overweight.
Shell defects	Shell-less, soft shell, cracked shell	Shell-less eggs are laid only with a membrane and no shell, soft shelled eggs have a very thin layer of shell deposited on the membrane while cracked eggs have various cracks from hairline cracks to large cracks in the egg. These defects could be as a result of immature shell gland, inadequate calcium or excess phosphorus in diet, heat stress and disease condition.
Texture	Corrugated, wrinkled, pimpled, broken and mended	These eggs are characterised by a rough texture which could take different forms including as a result of breakage and repair of the egg. They may be caused as

		a result of stress during calcification, poor lighting, disease such as infectious bronchitis, poor and inadequate nutrition, inability to control plumping and increase in age.
Calcium defects	Calcium coated, calcium deposits	These eggs have extra deposits of calcium on the eggs which can look like whitish deposits on the eggs. These defects could be as a result of defective shell glands, disruption during calcification and also excess calcium in the diet of the hens.
Structural defects	Mishapen eggs, white banded, slab-sided, double-yolked eggs	These are off shaped eggs that are either too large or too long. White banded, and slab-sided eggs are as a result of two eggs coming in contact in the shell gland. The first egg gets an extra coating of calcium which turns out whitish, while the second egg gets flattened at the point of contact. These defects can be as a result of stress, double ovulation (double yolked eggs), disease conditions, change in lighting and immature shell glands.

Source – (Roberts et al., 1998; USDA, 2000; Alltech, 2018),

1.4 Defence strategy of the hen and egg against microorganisms

1.4.1 – Contaminating microorganisms of importance in eggs

The presence of microorganisms plays an important role in egg production enterprises. In table eggs it affects its quality causing food borne gastroenteritis for consumers and public health concerns and in breeder eggs it affects its viability causing increased mortality of embryos, lower hatchability, and increased early chick mortality (Milakovic-Novak & Prukner, 1990). Several microorganisms are of economic importance to the egg industry including - *Bacillus cereus*, *Brucella*, *Campylobacter*, *Clostridium*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus*, *Vibrio* and *Yersinia enterocolitica*, however *Salmonella* is the most common, causing *salmonellosis* which affects up to 1.4 million human yearly in the United States (Voetsch et al., 2004). Over 2500 serovars of non-typhoidal *Salmonella* species are reported to be distributed in nature in soil, water, and animal hosts (Hellberg & Chu, 2016; WHO, 2018), however, *Salmonella enteritidis* and *Salmonella typhimurium* are the two most important serotypes of *Salmonella* transmitted from animals to humans in most parts of the world and can potentially cause severe diseases in the young, the elderly, and people with weakened immunity. An review of Braden (2006) reported that four *Salmonella* serotypes: *Salmonella typhimurium* (19%), *Salmonella enteritidis* (14%), *Salmonella newport* (9%) and *Salmonella javiana* (5%) accounts for almost one-half of all human isolates reported in the United States. *Salmonella typhimurium* has been reported to be strictly a human pathogen, causing invasive enteric fever, while most other *Salmonella* serotypes cause mainly gastrointestinal syndromes without bloodstream invasion.

Salmonella enteritidis is the second most commonly isolated serotype from human illness and is known to be strongly linked with shell eggs. In Europe, *Salmonella* infection of laying hens is dominated by *Salmonella Enteritidis* (EFSA, 2010) however, in Australia,

Salmonella Enteritidis has never been a national endemic in hen flocks, instead *Salmonella typhimurium*, often regarded as an external contaminant of eggs, was reported as the main cause of egg associated salmonellosis outbreaks (OzFoodNet Working Group, 2009). *Salmonella gallinarum* is also an important chicken pathogen, but rarely causes illness in humans. Some other serotypes of *Salmonella* are known to be associated mainly with other animals such as *choleraesuis* which affects swine, *dublin* (cattle) and *marina* (reptiles) (WHO, 2018).

Salmonella enteritidis has been the major cause of the food-borne salmonellosis pandemic in humans over the last 20 years, due to its ability to colonize the reproductive organs (Keller et al., 1997; Okamura et al., 2001). It is also of high importance to the egg industry because it has the unique ability to contaminate eggs without causing distinct illness in the birds infected (Guard-Petter, 2001). *Salmonella enteritidis* may also have a higher tropism and affinity for the reproductive organs of the hen than other serovars, and it persists longer in the blood after intravenous inoculation than other *Salmonella* serovars and may have virulence factors that enhance the chances to reach the ovary or oviduct in the course of an infection (Okamura et al., 2001; De Buck et al., 2004). Several studies have described the pathway of *Salmonella* infection in hens and the review by Wales & Davies (2011), summarises that it involves colonization, survival and multiplication of the pathogen in the host environment and finally penetration to the egg.

The chicken egg is a sterile entity however, it is susceptible to microbial invasion through various ways. Several studies, (Bichler et al., 1996; De Reu et al., 2006b) and reviews (Guard-Petter, 2001; De Buck et al., 2004; Gantois et al., 2009; Raspoet et al., 2011; Svobodová & Tómová, 2014) have reported on the types and mechanisms of egg contamination which can be either vertically (systemic contamination through the

reproductive organs of infected hens) or horizontally (shell surface contamination as a result of either infection of the lower reproductive tract or faecal contamination) as presented in Figure 1.6

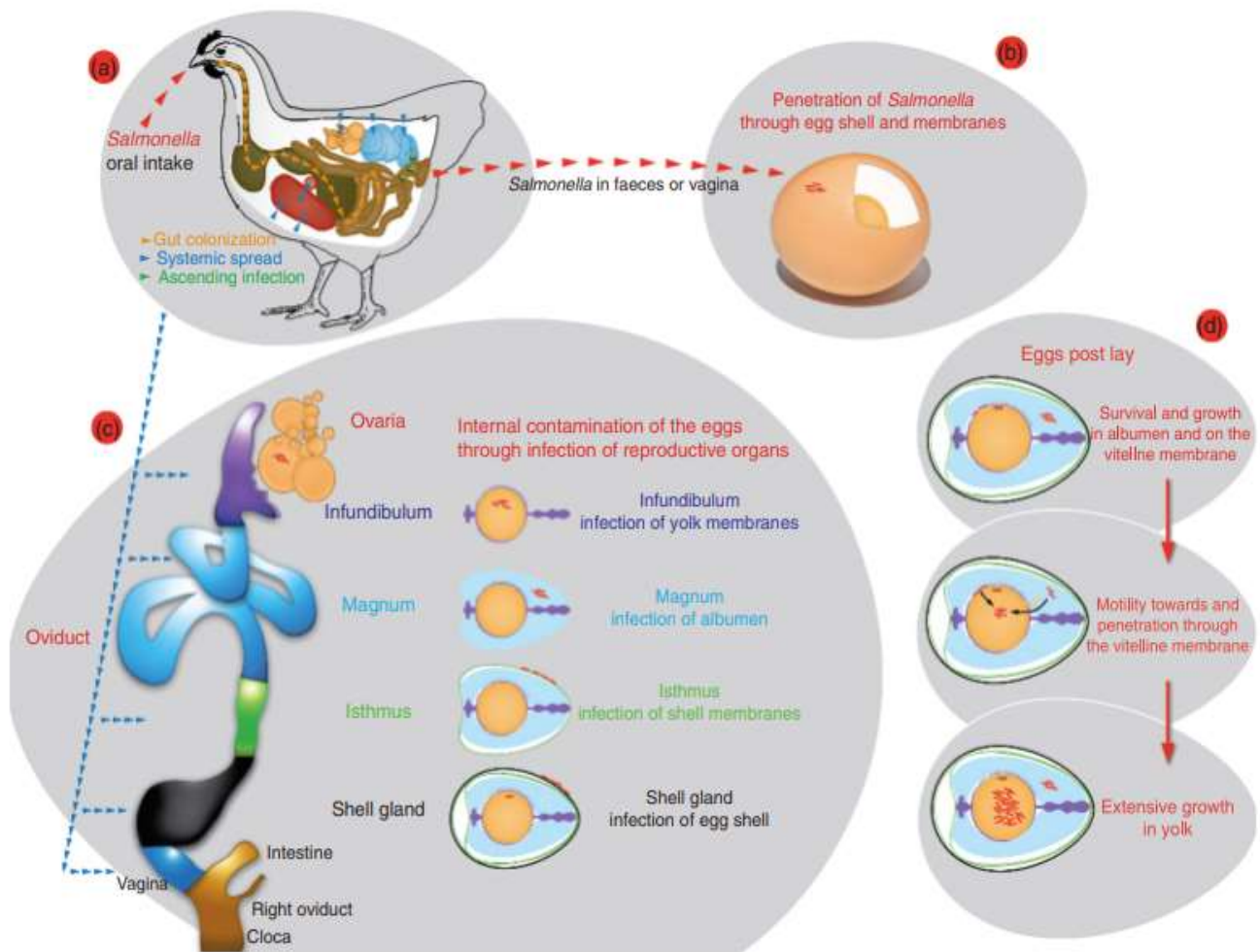


Figure 1.6 - Pathways of systemic or external *Salmonella* contamination of the egg. Image from Gantois et al., (2009).

Systemic contamination is the underlying phenomenon leading to the production of contaminated eggs (Howard et al., 2012). It occurs before the eggs are laid and can be as a result of infection of the reproductive organs of laying hens, leading to penetration of microorganisms through the eggshell in the shell glands, or by direct contamination of egg

contents within the oviduct. The findings of Thiagarajan et al (1994) and Dorairajan Thiagarajan et al (1996), reports that *Salmonella enteritidis* interacts with the granulosa cellular components of the preovulatory follicle in an invasion and multiplication manner, and spreads through the blood to the ovary (Timoney et al., 1989; Shivaprasad et al., 1990). The bacteria then penetrates the basement membrane (which terminates in blood vessels) (Griffin et al., 1971) and crosses the perivitelline layer into the yolk (Gast & Holt, 2001).

In healthy hens, external or shell surface contamination by excreta is rare because during oviposition, the stretching of the cloacal closes the intestinal tract and thus prevents contact with excreta. However, various studies have reported on the possibility of penetration and contamination of *Salmonella* (Wang & Slavik, 1998; Miyamoto et al., 1998; Berrang et al., 1998; Berrang et al., 1999) and *Escherichia coli* (Haigh & Betts, 1991) after oviposition. This is largely caused through pores or cracks in the shell, faecal contact of the egg by the hen, and poor hygiene conditions of the hen housing or nest boxes, air, handlers, and processors. To control the spread of *Salmonella* contamination in hen and eggs, the study of Hassan & Curtiss (1997) and Gantois et al (2006) reports that vaccination with live avirulent *Salmonella* can induce long-term protection against infection in layers. Other interventions aimed at preventing the contamination and spread of *Salmonella enteritidis* from farm to plate include farm-based programs such as enhanced biosecurity which can help control the bacteria in young laying hens, immediate and continued refrigeration of eggs (Gast & Holt, 2001), education of consumers and food handlers on the potential and risk of *Salmonella* in raw and uncooked foods (Braden, 2006) and the use of prebiotics, probiotics, symbiotics and other feed additives to increase the resistance of the hen against infections (Van Immerseel et al., 2002).

1.4.2 - Natural resistance of the egg against microbial contamination

As shown in Figure 1.7, the egg is designed to protect itself from bacterial invasion. Microorganisms are thus faced with several innate defence structures (cuticle, eggshell, shell membranes, albumen, chalazae, vitelline membranes - all which serve to protect the yolk from bacterial invasion) before external microbial contamination can take place.

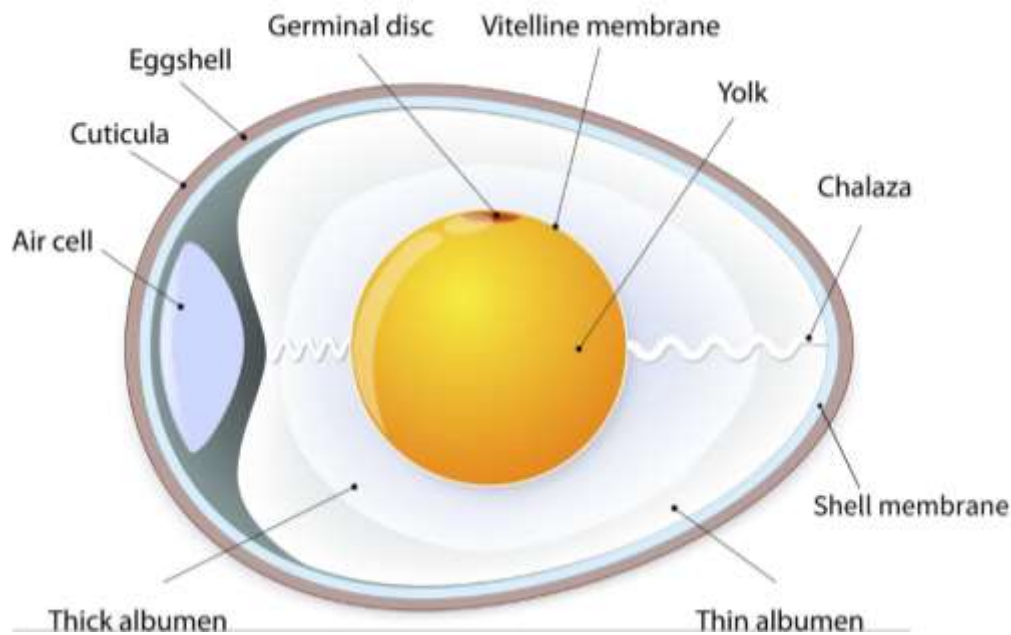


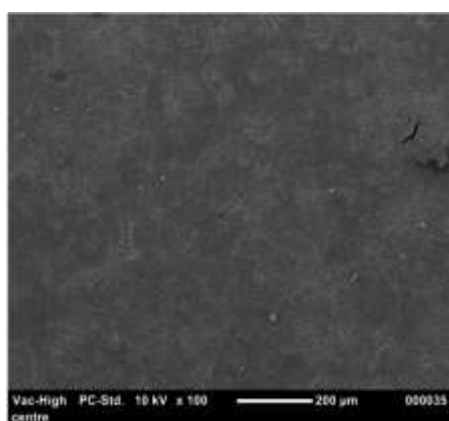
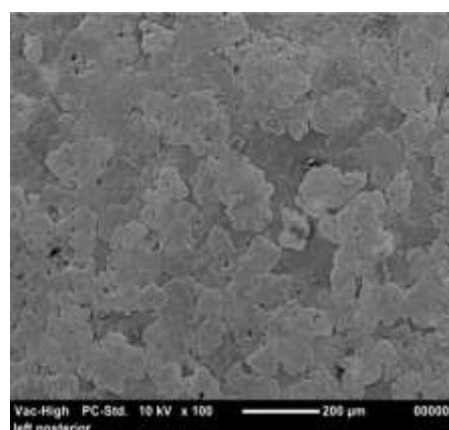
Figure 1.7 - Schematic representation of the egg structure. Source (Hill, 2019)

1.4.2.1 – The cuticle

The cuticle is the outermost layer of the eggshell and is an important defence structure of the egg. With a diameter of about 12 μm , it defends the egg by adding to the thickness of the shell, prevents moisture loss from within the egg and protects the egg from external microorganisms (De Reu et al., 2006b). It is deposited on the vertical crystal layer of the eggshell, in the palisade layer of the eggshell during the last 1.5 – 2 hr of eggshell formation in utero (Baker & Balch, 1962). The structure, constituents, functions, and synthesis of the cuticle, as well as the effect of age, washing and shell colour on the strength of the cuticle have been excellent reviewed by (Samiullah & Roberts, 2014). The cuticle is made up of inner calcified and outer non-calcified water insoluble layers which are composed of

glycoproteins such as ovocalyxin and ovocleidin (Bain et al., 2013), lysozyme C, mucin, ovotransferrin as well as some carbohydrate and fat constituents. The study of Chen et al., (2021) reported that the proteome of eggs ranked as having good quality cuticle had Adrenomedullin (ADM), Phospholipase C gamma 2 (PLCG2), Prolactin receptor (PRLR) and Prostaglandin D2 synthase (PTGDS) differentially expressed compared to eggs ranked as poor quality cuticle. The cuticle of a freshly laid egg is viscous and less effective in resisting bacterial penetration as it is immature. The cuticle dries within six minutes of lay (Sparks & Board, 1985) and after about 6 hours, it consolidates and matures. After about 96 hours, it begins to crack. The study of Muñoz et al (2015) reports that the cuticle is most effective against bacterial penetration between 6 and 72 h after eggs have being laid, when the coating is fully mature but has not dried excessively. An excessively dried cuticle compromises its function as water and bacteria can move more easily through the cuticle crevices and gain access to the egg interior. Cuticle deposition on the eggshell is influenced by hen age, with eggs from younger hens having thicker cuticle compared to older hens (Lunam & Ruiz, 2000; Rodríguez-Navarro et al., 2013). The breed of the hen is reported to have no effect on the cuticle quality because shell pigmentation is done at the basal end of the shell gland before the egg is moved into the vagina for blooming or cuticle deposition. In addition, the work of Samiullah & Roberts, (2013) reported that protoporphyrin is located in the calcareous part of the eggshell (80 – 87%) with a minority contained within the cuticle (13-20%). Protoporphyrin gives most of the colour to brown hen eggshells and is reported to be antimicrobial against gram positive bacteria. Investigation of the ultrastructure of the cuticle showed that it is granular, vesicular, homogeneous, porous and layered with air inclusions (Simons & Wiertz, 1966). The research by Samiullah & Roberts (2013) scored cuticle as good and intact cuticle if it had a coverage of 91 – 100%, less patchy cuticle when having 61 – 90 % coverage, more patchy with 11 – 60 % coverage and negligible or no

cuticle with less than 10% coverage. The structure of the cuticle is fairly stable, however, as shown in Figure 1.8, washing (Lorenz & Starr, 1952; Simons & Wiertz, 1966; Samiullah et al., 2013; Liu et al., 2016), has been reported to damage the cuticle as it partially or completely removes the layers thereby exposing cracks and pores which can lead to bacterial penetration and contamination. Contrarily, the study by Leleu et al (2011) reported no changes to the cuticle quality of eggs from hens in the late laying stage. According to the studies of Simons & Wiertz (1966), Liu et al (2016) and the review of Howard et al (2012), cuticle structure can also be affected by abrasion, temperature and humidity of the environment after oviposition and during storage, as well as storage time.



Scanning electron microscope image of eggshell with intact cuticle in unwashed eggs. Scanning electron microscope image of eggshell with patchy cuticle in washed eggs.

Figure 1.8 - Scanning electron microscope images of cuticle of washed and unwashed eggs.

Source: Samiullah & Roberts, (2013).

1.4.2.2 – The shell and shell membrane

The eggshell is an important antimicrobial defence structure of the egg. It provides a container for the egg's constituents, mechanical protection against environmental shocks and damage, and serves as a channel for controlled gas exchange. Several researchers have studied and reviewed the formation, ultrastructure, crystallographic texture, mineralisation

and proteomics composition of the hen eggshell complex (Stewart, 1935; Solomon et al., 1994; Panheleux et al., 1999; Hunton, 2005; Nys & Guyot, 2011; Hincke et al., 2011; Hincke et al., 2012; Rahman, 2013; Svobodová & Tůmová, 2014; Du et al., 2015; Kaspers, 2016; Mishra et al., 2020; Chen et al., 2021). The shell of the egg is made up of morphologically distinct sections which are sequentially added in the isthmus and uterine segments during its passage along the oviduct. The first layer is the shell membranes, which are two fibrillar membranes which are proteinaceous and disulfide-rich in nature and covers the egg white. The inner and outer shell membranes are joined by inter-connecting fibres that form a highly cross-linked fibrous meshwork, arranged in alternating layers parallel to the eggshell surface. Following the shell membranes is the mammilla knob layer which are mineralized discrete aggregations of organic material. This is the site of the shell matrix attachment and where calcium carbonate crystallisation takes place. The third layer is the hard – shell fibrous complex layer also known as the palisade, and lastly, the leathery cuticle which is the most external layer of the eggshell. The study by Lifshitz et al (1964) reported that the inner membrane is the most effective compared to the shell and outer membrane to prevent bacterial entry. This is due to the tighter meshwork of the inner membrane compared to the outer membrane. The study by Nys et al (2001) reported on the antimicrobial component of the eggshell matrix proteins. They concluded that eggshell has proteins – ovocleidins – 17 and –116 and ovocalyxins – 32 and –36), which play functional roles in regulation of eggshell mineralization and anti – microbial defence. The review by Hincke et al (2012) also documented that the eggshell has over 500 proteins identified by mass spectrometry and egg white proteins – ovalbumin, lysozyme and ovotransferrin are also present in the uterine fluid, and are primarily localized in the innermost regions (shell membranes and mammillary cone layer) of the eggshell. Figure 1.9 describes the distribution of eggshell proteins.

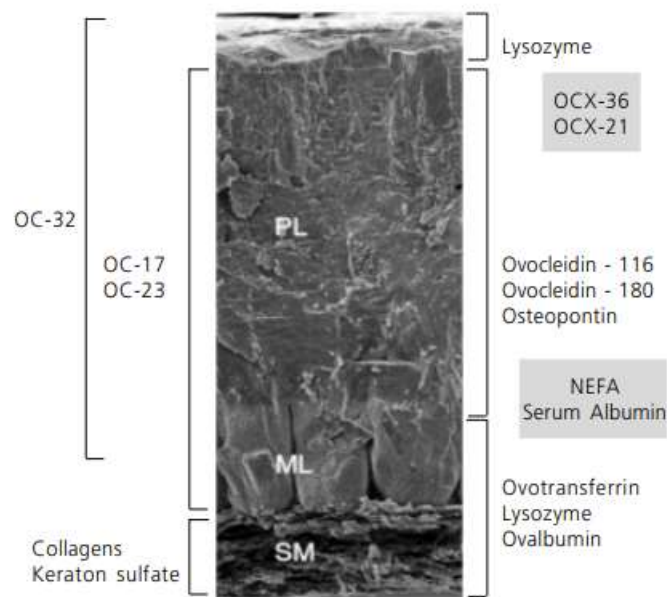


Figure 1.9 - Distribution of eggshell proteins. Personal communication of Dr. Max Hincke, from the University of Ottawa.

1.4.2.3 – Egg albumen

The albumen of the hen egg has been described to be in four distinct layers (Almquist & Lorenz, 1933; Asmundson & Burmester, 1936; Sturkie & Polin, 1954). The first three layers; chalaziferous layer (attached at the poles of the yolk), inner liquid layer (surrounding the yolk), and firm layer (which makes up 50–60 percent of the albumen), and last layer, that is the outer liquid layer, which makes up one fourth of the total albumen and is secreted by the isthmus and uterus (Svobodová & Tómová, 2014). The early studies by Surface (1912), Bradley (1928), Asmundson & Burmester (1936) and Cole (1938) reported that the magnum – the upper part of the oviduct – is made up of tubular and unicellular glands. They also reported that the proteins of the albumen are formed in the magnum and in hens which produce a more viscous albumen, the goblet cells of the unicellular glands were seen to be more developed compared to hens that produce less viscous inner thick albumen. It has been established that the growth and multiplication of microorganisms is slowed due to the

viscosity, pH and antibacterial nature of the albumen proteins such as mucin, lysozyme and conalbumin (Mayes & Takeballi, 1983). This may suggest that having hens with a more developed goblet cells of the unicellular glands will lead to the production of more viscous thick albumen and hence better protection against bacterial invasion.

1.4.2.4 – Vitelline membrane

The vitelline membrane is an important structure of the egg which surrounds the yolk and plays multiple roles including maintaining the structure of the yolk, separating the contents of the yolk from diffusing into the albumen as well as preventing moisture content from penetrating into the yolk from the albumen. Equally importantly, the vitelline membrane provides protection against bacterial invasion that made it into the albumen. The study of Moran & Hale (1936) found that the vitelline membrane consists of three layer which can be separated using the Chambers micro-manipulator. The first layer which is nearest to the yolk has a thickness of 3 – 6 μ . The middle layer has a thickness of 6 – 10 μ while the outer layer has a thickness of 3 – 6 μ . The middle layer of the membrane is reported to be made up of keratin and formed from a part of the membrane which lines the follicular wall of the ovum. The outer layer however is made of mucin secretions from the infundibulum which are precipitated on the yolk to form the thickened and strengthened collagenous membrane covered with a layer of mucin (McNally, 1943). The yolk is the life of the egg and contains nutrients that are desirable for the growth of bacteria. The albumen on the other hand contains antimicrobial peptides that engulfs bacteria and further protects the yolk from bacterial invasion. Proteomic analysis of the chicken egg vitelline membrane showed that the vitelline outer membrane protein (VMO) VMO II was identified as a β -defensin-11 (Mann, 2008). Most defensins have showed antimicrobial activity against a wide range of pathogens including bacteria and fungi. Avian β -defensins are also important as they support the innate

defence system because avian heterophil lacks oxidative mechanisms. Another study investigated the potential of vitelline membrane outer layer protein 1 against *Listeria monocytogenes* and/or *Salmonella* and reported that it had additional structural features that can contribute to its antimicrobial potential (Guyot et al., 2016). The vitelline membrane is affected by storage time and conditions. As the egg ages, the albumen pH increases leading to the breakdown of the gel characteristics of the albumen. This increases moisture contacts with the vitelline membrane and increases dissipation of the network of fibres on the surface of the membrane, thus weakening it (Fromm, 1967). A weak vitelline membrane leads to the flattening of the yolk and a demonstration of loss in yolk quality. An extreme weakening or breakage of the yolk membranes can lead to the contents of the yolk diffusing and mixing with the albumen which inhibits the antimicrobial properties of the albumen (Chen et al., 2005; Caudill et al., 2010; Bratanov et al., 2011). Age of the hen also affects the strength of the vitelline membrane. The study of Marzec et al (2019) reported that membranes from the yolks of older hens (aged 45–70 weeks of age) was weaker than from younger hens aged 25–26 weeks by 18%.

1.4.2.5 – Chalazae

The chalazae have been described by Romanoff & Romanoff (1949), as ropy structures of cloudy material attached at both ends of the egg and made up of many fine mucin-like fibres (similar to the structural component of the thick albumen) that spiral from the yolk into the albumen. The studies of Itoh et al (1987) indicated that that ovomucin and chalaza were made up of almost the same basic units and the chalaza may be a higher polymerized state of ovomucin. Studies have shown that the chalazae are formed as a result of suspension of mucin fibres by the rotation of the egg. The elements of the chalazae is differentiated from the other components of the eggs in the posterior division of the magnum, the isthmus and uterus

(Hansen, 1933; Almquist & Lorenz, 1933; Asmundson & Burmester, 1936; Scott & Huang, 1941). The infundibular chalaza which extends from the large end of the egg is composed of a single strand of fibres and has a clockwise twist while the cloacal chalaza extends at the small end is longer and larger, consist of two heavy strands and twisted in a counter - clockwise pattern (Almquist, 1936; Baker & Stadelman, 1958). One of the measures used in grading eggs for quality is the positioning and movement of the yolk within the egg. The cords of the chalazae play an important role anchoring and maintaining the yolk at the centre of the egg so as to ensure maximum coverage and protection of the yolk by the albumen against physical shocks and antimicrobial invasion. The yolk contents are largely lipids and thus light in weight. Without the chalazae, the yolk could slip out of the gelatinous thick albumen towards the shell where it gets more exposed to physical damage and bacterial invasion. The presence and prominence of the chalazae is a feature of consumer preference with majority preferring eggs with less prominent chalazae. It is thus important to note and educate consumers against the misinformation that the chalazae are a foreign material in the egg, or a sign of fertilization of the egg. The study of Baker & Stadelman (1958) documented that the prominence of the chalazae is a heritable trait which varies among strains. They also reported that there was no association between chalazae prominence and internal egg quality and the prominence of the chalazae did not deteriorate like the rest of the egg with increasing storage time and environmental conditions.

1.5 Conclusion

The world's population is projected to increase by 25% from 7.8 billion in 2022 to 9.8 billion in 2050. To meet this challenge, global food production is estimated to increase by 60%. Eggs constitute one of the most affordable sources of animal protein, and are rich in vitamin A, folate, biotin and iodine, and vitamin D. They are also rich in antioxidants which have major benefits for health and wellbeing. The outstanding nutrient profile of the hen egg and other egg products has resulted in its high demand by egg consumers. In addition, the short generational interval of the hen, relatively quick returns on investment for egg producers has made layer production to be recognized as a reliable intervention strategy to improve human nutrition, end hunger and food insecurity and reduce poverty. Taken together, this has heightened the investments in global poultry research, causing immense genetic improvement by poultry breeders and geneticists and necessitated corresponding management actions by egg producers. All these are targeted to optimise egg production in a sustainable and profitable manner, maintain uniformity of performance traits in flocks and increase feed to egg conversion efficiency, albeit maintaining quality of product.

Due to genetic improvement of the hen, egg production has increased from 150 eggs per hen per year in the early 1900s to around 300 eggs per hen annually in the 2000s. However, with this increase comes the tendency of increased feed intake and hen bodyweight, compromised egg quality, higher metabolic rates, and predisposition to increased body heat, which causes physiological stress and other welfare and health concerns for the hen. The egg industry therefore needs advanced strategies to maintain uniformity, enhance production efficiencies, increase product quality and farmers profitability, meet hen needs and welfare, and satisfy consumers preferences. These improvements could be achieved through changes in hen management approaches, advanced selections and genetic improvement of performance traits, and improved use of technology.

1.6 Thesis aims and objectives

The overarching aim of this thesis was to characterise the variation in animal performance and egg quality between laying hens and evaluate a dietary intervention to improve uniformity.

The specific objectives of this research were to:

- a. Investigate the inherent variation and associations in hen performance traits (feed intake, egg production, and body weight changes) and egg quality measurements (albumen, yolk, and shell characteristics) of individual early laying ISA Brown hens aged between 25 to 30 weeks old.
- b. Evaluate the effect of feed efficiency on body weight, egg production, nutrient digestibility, and egg quality characteristics of individual ISA Brown hens in the mid laying stage of egg production (35 to 40 weeks), ranked based on their FE obtained during the early lay period (25 to 30 weeks).
- c. Evaluate the effect of feed efficiency and body weight on organ characteristics and incidences of fatty liver haemorrhagic syndrome of hens in the mid stages of lay.
- d. Determine the differences in the bacteriostatic properties of egg albumen from late laying individually housed ISA Brown hens with varying feed efficiencies.
- e. Explore the differences in the proteome composition of egg albumen from late laying individually housed ISA Brown hens with varying feed efficiencies.
- f. Evaluate the effect of quantitative restricted feeding techniques on hen performance traits, egg quality and organ characteristics of individually housed hens.

1.7 Significance of the research

- a. This study supplies novel knowledge on the onset, extent and persistence of variation in hen performance and egg quality traits of individual hens. Also, it furnishes scientific literature with the relationships that exist in performance traits within individual hens in a flock. This will be useful for egg researchers for comparison and references, as well as to serve as a building block for further research to investigate strategies that may improve uniformity in hen performance, egg quality and hen welfare traits.
- b. The findings from this research will be useful for egg producers towards improving the economics of hen and egg production and improving management decision making.
- c. The outcomes from this research may also be important for egg breeders by providing information on performance traits that merit advanced study for improved selection towards increased productivity.

1.8 Thesis questions

The following are the questions which were answered in the research contained in this thesis.

- a. What is the extent of variation for common production variables such as feed intake, bodyweight, egg weight, egg mass and feed efficiency, and egg quality traits among individual laying hens and how early in lay do these variations set in?
- b. How does variation in feed efficiency affect body weight, egg production, nutrient digestibility, and egg quality of individual hens in the mid stages of lay?
- c. How does variation in feed efficiency influence organ characteristics and incidences of fatty liver haemorrhagic syndrome in mid laying ISA brown hens?
- d. How does varying feed efficiencies influence the bacteriostatic property of egg albumen from ISA Brown hens? Are there differences in the proteome of egg albumen from ISA Brown hens of varying feed efficiencies?
- e. How does a feed intervention strategy affect hen body weight, egg production, feed efficiency, abdominal fat pad deposition, organ characteristics and incidences of fatty liver haemorrhagic lesion syndrome in individual ISA brown hens?

CHAPTER TWO

2. Variation and association of hen performance and egg quality traits in individual early – laying ISA brown hens

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This is the first result chapter of this thesis, and it investigates the inherent variation and associations in hen performance traits (feed intake, egg production, and body weight changes) and egg quality measurements (albumen, yolk, and shell characteristics) of individually housed hens in the early stages of lay. In this study, we hypothesised that there are variations in hen performance and egg quality traits of individually housed hens in early lay, kept under the same management conditions.

2.1 Abstract

Uniformity in hen and egg traits is an important consideration in commercial layer flocks, however, there is little information on variation in hen performance traits and how it affects egg quality variables. This study investigated the presence and extent of variation in performance traits of individual hens and its associations with egg quality characteristics. A total of 450 ISA Brown hens were individually housed and monitored for 42 days from 25 to 30 weeks of age, to measure hen performance characteristics. Forty-four hens from the flock were randomly selected and eggs were collected from the same hen once weekly for albumen, yolk, and shell assessments. The means $\pm$ standard deviation of average daily feed intake (ADFI), albumen height, initial body weight (IBW), and final body weight (FBW) were $124 \text{ g} \pm 15$, $10.3 \text{ mm} \pm 1.5$, $1802 \text{ g} \pm 129$, and $2000 \text{ g} \pm 175$, respectively. Albumen height was not associated with ADFI ($r = 0.18$, $p = 0.21$), IBW ($r = -0.04$, $p = 0.79$), or FBW ($r = -0.06$, $p = 0.69$). This study showed that variation exists in feed intake, body weight, and albumen quality of individual hen in early lay stage and an increase in feed intake and body weight did not influence albumen quality.

Keywords: albumen quality; laying hens; feed intake; body weight; egg production

2.2 Introduction

The average daily feed intake (ADFI), bodyweight (BW), egg weight (EW), and egg production (EP) of the hen, as well as the albumen, yolk, and shell indices of the egg, are important measures of productivity in the layer industry. Within modern layer strains and production systems, these traits are influenced by various environmental factors such as diet, rearing systems, and disease status (Williams, 1992; Roberts, 2004) which lead up to the onset of lay and beyond. In commercial laying flocks, ADFI and EP are difficult to be measured on an individual hen basis, thus concealing the extent of variation and possible associations linked with egg quality. This may influence management decision making towards achieving optimum productivity in the flock. Previous research has explored hen performance traits and/or egg quality on a flock basis (Şekeroğlu & Altuntaş, 2009) or on white-shelled hens (Lacin et al., 2008), however, the extent of variation in performance traits of individual hens and their association with egg quality of the same hens merits investigation (Tu et al., 2011).

Hen BW is an important contributing factor to feed intake, attaining sexual maturation and egg weight (Harms et al., 1982; Bish et al., 1985). Under certain production settings where feed allocation cannot be readily controlled, the BW of hens has been reported to surpass the breed standard recommendation (Parkinson, Cransberg, and Fadavi-Firooz, 2007). Excessive body weight can result in lack of laying persistency, production of larger eggs causing lower eggshell quality (Parkinson, Cransberg, and Fadavi-Firooz, 2007; Parkinson, Roberts and Horn, 2015), increased fat accumulation causing multiple ovulations and leg health problems (De Beer & Coon, 2005). Reports on the variation and associations between hen performance and egg quality are equally scarce in the literature. In a study with white-shelled hens ranked by BW, Lacin et al., (2008), reported that light BW hens had a higher EP, laid bigger eggs, had a lower feed conversion ratio (FCR), and had higher albumen heights compared to the

heavy BW birds. Akter et al., (2018) also reported associations between performance traits such as ADFI, egg weight and BW, with egg quality characteristics in mid-lay ISA Brown layers, where hens had varying feed intake but comparable egg mass. Akter's study also showed that hens with a low (better) FCR had a higher albumen height and their yolks had lower levels of lipid peroxidation markers. The implication of that study was that ADFI, BW and feed efficiency might be associated with indices of egg quality, however, it was conducted on 55 – week – old hens, thus, there are no indications of how early in lay such variation and associations arise. Therefore, the aims of this study were to investigate the inherent variation in hen performance traits (feed intake, EP, and BW changes) and their associations with egg quality measurements (albumen, yolk, and shell characteristics) of individually housed ISA Brown hens during the early stages lay.

2.3 Methodology

Animal Ethics

The experiment was conducted at the Poultry Research Foundation (PRF) layer rearing facility, within The University of Sydney Camden Campus, New South Wales, Australia, in accordance with the 8th edition of the Australian code for the care and use of animals for scientific and experimental purposes (NHMRC, 2013). The procedures and activities conducted in this study, were reviewed, and approved by the University of Sydney Animal Ethics Committee.

Animal management and experimental design

A total of 455, 21-week-old ISA Brown pullets were weighed individually and randomly distributed to 25 × 50 × 50 cm single metal layer cages. Shed temperature and photoperiod were managed according to the ISA Brown management guide (ISA Brown, 2017). A wheat/sorghum and soybean meal mash diet formulated to supply 2750 kcal/kg gross metabolizable energy and 16.30% crude protein was offered to the birds on an *ad libitum* basis in individual metal feeders. The experimental diet as presented in Table 2.1, met the National Research Council requirements for ISA Brown laying hens in early lay. (NRC, 1994). Hens were provided with water *ad libitum* via automatic nipples in each individual cage. At the end of the experiment, data from nine hens were excluded from the data set because three out of the nine hens died prior to the end of the experimental period, while the remaining six had an average EP of 7%.

Production performance

At 25 weeks of age, data collection of hen performance variables began and lasted for six weeks until 30 weeks. This age of hens was chosen because hen performance and egg quality are known to change with hen age, thus it is important to determine how early in lay these

differences and changes begin. Further, hens tend start to lay eggs between 18 and 24 weeks and reach peak production at about 25 weeks. The investigation thus ensured that all hens had reached peak production but were still in the early stages of lay. Individual BW of the hens was taken at 21 weeks (initial body weight - IBW) and 30 weeks (final body weight - FBW) and body weight change, BWC were calculated as the difference between IBW and FBW. ADFI was calculated for individual hens as feed offered - feed uneaten / 7 (number of days in the week). EP was recorded daily and calculated in percentages (total number of eggs produced in seven days $\times 100$) / 7 (number of days of the week). Egg collection was done twice daily, and eggs were weighed using an electronic scale with a digital output. Egg mass (EM) per hen per day was calculated as $(EP \times EW) \div 7$, where EP = egg production and EW = egg weight. Feed conversion ratio (FCR) was calculated on a weekly basis as grams of feed consumed per gram of EM for each hen.

Table 2.1 - Dietary composition of basal diet fed to experimental hens.

Feed Ingredient	Amount (kg/tonne)
Wheat 10.5%	346.80
Sorghum 12%	345.00
Soybean meal 46%	155.00
Limestone grit 38%	71.00
Canola expel 32%	30.00
Limestone	20.00
Dicalcium phosphate	15.00
Soybean oil	7.00
Sodium bicarbonate	3.19
DL-methionine	1.75
Lysine-HCl	1.70
Salt	1.60
Layer premix*	1.00
L-Threonine	0.45
Choline chloride 60%	0.30
Ronozyme WX CT (DCM)	0.12
Ronozyme Hi-phosphate layer 300 (DCM)	0.03
Total	1000.00
Calculated Nutrient Analysis	
Crude protein (%)	16.30
Gross metabolizable energy (kcal/kg)	2750.00
Net energy (kcal/kg)	2106.00
Crude fat (%)	2.90
Total digestible lysine (%)	0.74
Total digestible methionine (%)	0.39
Total digestible tryptophan (%)	0.18
Total digestible isoleucine (%)	0.58
Total digestible arginine (%)	0.83
Total digestible valine (%)	0.65
Calcium (%)	4.00
Linoleic acid (%)	1.39
Crude ash (%)	13.40
Sodium	0.18
Lysine (%)	0.82
Total Phosphorus (p) (%)	0.61
Available (p)	0.40
Methionine (%)	0.42
Xanthophyll (mg/kg)	6.00

*each kilogram contained vitamin A 15 000 IU; cholecalciferol 1 500 ICU; dl- α -tocopheryl acetate 30 IU; menadione 5.0 mg; thiamine 3.0 mg; riboflavin 6.0 mg; niacin 20.0 mg; pantothenic acid 8.0 mg; pyridoxine 5.0 mg; folic acid 1.0 mg; vitamin B1 15 μ g; M 80.0 mg; Z 60.0 mg; F 30.0 mg; Cu 5.0 mg; 2.0 mg; and Se 0.15 mg

Egg quality assessment

A total of 44 hens were randomly subsampled from the flock and once weekly, eggs were collected from the same hens for assessment of albumen, yolk, and shell characteristics. After collection, eggs were weighed individually using an electronic balance with a digital readout, to the precision of 0.01 g. Egg height (mm) and width (mm) were measured, using a 200 mm electronic Vernier calliper (Kincrome, Australia). Egg shape index was calculated as egg width/ egg height $\times 100$. The eggs were carefully broken out on a flat, levelled glass surface on a metal stand with reflective mirror. For the broken-out eggs, the thick albumen and yolk widths were measured with a digital vernier calliper at the equator while still combined. Albumen height was measured using an albumen height gauge with an electronic display (Technical Services and Supplies, York, United Kingdom). The probe on the gauge detects and measures the height of the thick albumen when an egg is broken out onto a flat surface. Using a plastic scraper, the albumen was separated from the yolk, and collected into a clean container and weighed. The yolk height was measured using an AMES tripod micrometre (Waltham, USA) and yolk colour determined using a DSM colour yolk fan, (DSM - Switzerland 2005), on a scale from 1 through 16 units (0 being pale yellow and 16 being red-orange). The yolk was collected in a clean container and weighed using an electronic balance with a digital readout. The pH of the albumen and the yolk were measured with a pH meter (Electronic Instrument Ltd). The pH meter was first standardised using buffer solution of pH 4.01 and 9.20. The electrode was then rinsed with de-ionised water and dipped into the sample allowing sufficient time for stabilisation before taking the reading. The eggshells were carefully washed, air dried for 72 hours and weighed with a digital scale, to a precision of 0.0001 g. Eggshell membranes were removed, and eggshell thickness (mm) was measured at three regions (pointy end, equator, and blunt end) using a digital calliper, to the nearest 0.001 mm; the mean obtained from the three values was recorded as the average shell

thickness. The eggshell percentage was calculated as the eggshell weight multiplied by 100 and divided by the egg weight. The Haugh unit, was derived using the formula $100 \times \log_{10}(h - 1.7 \times w^{0.37} + 7.6)$, where h = albumen height (mm), and w = egg weight (g) (Şekeroğlu & Altuntaş, 2009). The albumen and yolk ratios were calculated from the individual weights of the albumen and yolk. Yolk index was calculated as yolk height/ yolk width $\times 100$, and albumen index was calculated as albumen height/ albumen width $\times 100$. Fresh eggs were collected from the same hens the following day and eggshell breaking strength (g), was measured using a texture analyser (Perten TVT 6700, Stockholm - Sweden), fitted with a cylindrical probe 75 mm in diameter, as the peak force which must be applied to the egg before it breaks.

Statistical analysis

Descriptive statistics including means, standard deviation, and coefficient of variation of hen performance and egg quality data were analysed using SAS[®] University Edition software. Repeated measures data were analysed using the MIXED Procedure of SAS to assess between week differences. Pearson or Spearman (for yolk colour) correlation coefficients for egg quality measurements were generated using the CORR Procedure in SAS. Differences between weekly means and correlation coefficients values were considered significant if the p value was ≤ 0.05 .

2.4 Results

Hen performance

The summary statistics of individual hen performance between 25 and 30 weeks of age are shown in Table 2.2. The hens had an average IBW of 1803 g $\pm$ 129 (CV = 7%) at 21 weeks and FBW of 2000 g $\pm$ 175 (CV = 9%) at 30 weeks. The ADFI had a CV of 12% while egg weight had a CV of 6%.

Table 2.2 - Descriptive statistics of hen performance of 446 individually caged ISA Brown hens aged between 25 and 30 weeks old.

25 to 30 Weeks							
Performance Trait	<i>n</i>	Mean	Min	Max	SD	CV (%)	BS
Eggs produced per hen/week (%)	2676	98	0	100	6.8	7.0	95.6
Egg weight (g/day)	2676	61	48	78	3.8	6.2	61.7
Egg mass (g/day)	2676	59	0	77	6.3	11	58.9
Average daily feed intake (g/day)	2676	124	60	191	15	12	114
Feed conversion ratio	2676	2.1	1.0	3.4	0.2	12	1.93
Initial body weight, 21 weeks (g)	446	1803	1434	2342	129	7.2	1660
Final body weight, 30 weeks (g)	446	2000	1476	2668	175	8.8	1835
Body weight change (g)	446	198	-194	522	103	52	-

SD: standard deviation; CV: coefficient of variation; Min: minimum; Max: maximum; BS: Breed standard

The distribution of hen performance traits is shown in Figure 3.1. A total of 80.9% of the hens in the flock consumed more than 114 g of feed as recommended by the ISA brown breed guide. The initial and final body weight of 88% and 82% of the flock was above the breed

expectation of 1660 g and 1835 g respectively. The feed conversion ratio of 72% of the flock was above 1.93 while 48.9% of the flock laid eggs with weights below the breed standard.

The weekly means of hen performance traits are presented in Table 2.3. Average daily feed intake increased significantly from 126 g at 25 weeks to 128 g at 30 weeks ($p < 0.0001$), however there was a significant and unexplainable drop in feed intake in week 28. Egg weight also increased significantly from 60 g to 63 g between 25 and 30 weeks of age ($p < 0.0001$).

Table 2.3 - Changes in mean hen performance traits of individually caged ISA Brown hens between 25 and 30 weeks of age (n = 2676).

Parameters	Age of Hen (Weeks)						SEM	p-Value
	25	26	27	28	29	30		
Egg produced per hen per day (%)	98.6 ^c	98.1 ^{ac}	98.3 ^{ac}	97.6 ^{ab}	97.2 ^b	98.5 ^c	0.32	0.012
Egg weight (g)	60 ^b	61 ^c	62 ^a	62 ^a	62 ^a	63 ^d	0.18	< 0.001
Egg mass (g)	58 ^b	59 ^{bc}	60 ^{ad}	59 ^{ac}	59 ^{bc}	60 ^d	0.29	< 0.001
Average daily feed intake (g)	126 ^{ab}	126 ^{ab}	125 ^a	117 ^c	123 ^d	128 ^b	0.70	< 0.001
FCR	2.17 ^a	2.13 ^{ab}	2.08 ^c	1.98 ^d	2.08 ^e	2.13 ^{bc}	0.01	< 0.001

FCR – Feed conversion ratio; SEM: standard error of mean; means with different superscripts are significantly different ($P < 0.05$).

The correlations between individual hen performance traits are described in Table 2.4. The ADFI was positively associated with all BW parameters ($p < 0.001$) and had a significant but weak positive association with EW ($r = 0.32$; $p < 0.001$). There was no significant association seen between EP and ADFI nor between EP and body weight parameters.

Table 2.4 - Correlation (r) among hen performance traits of early-laying ISA Brown hens aged between 25 and 30 weeks old; n = 446 hens.

Parameters	EP	EW	ADFI	IBW	FBW	BWC	FCR
EP	1						
EW	-0.03	1					
ADFI	0.07	0.32***	1				
IBW (21 weeks)	-0.10	0.36***	0.50***	1			
FBW (30 weeks)	0.01	0.28***	0.63***	0.81***	1		
BWC	0.15*	0.03	0.45***	0.12	0.68***	1	
FCR	-0.66***	-0.15*	0.5***	0.27***	0.32***	0.2**	1

*** coefficients with a *P* value < 0.0001; ** coefficients with a *P* value < 0.01; * coefficients with a *P* value of ≤ 0.05 ; BWC: body weight change; EW: egg weight; IBW: initial body weight; FBW: final body weight; ADFI: average daily feed intake; EP: egg produced per hen/day; FCR: feed conversion ratio; hen-day egg production (weekly) = total number of eggs/7 $\times$ 100; egg mass = (egg produced per hen/day (%)) $\times$ average egg weight (g)/100)

Egg quality

The descriptive statistics of egg quality characteristics are shown in Table 2.5. Briefly, the mean and standard deviation of albumen height was 11 mm $\pm$ 1.5, albumen weight was 37 g $\pm$ 3.0, yolk weight was 17.0 g $\pm$ 0.7, and eggshell weight was 6.1 g $\pm$ 0.6. The CVs for albumen height, albumen weight, yolk weight, and shell weight were 14%, 8%, 4%, and 10%, respectively.

Table 2.5 - Descriptive statistics of egg quality indices of 44 individually caged ISA Brown hens aged between 25 and 30 weeks old (n = 264).

Egg Quality Trait	Mean	Min	Max	SD	CV (%)
Egg height (mm)	55	51	60	1.7	3.1
Egg width (mm)	44	42	57	1.3	2.9
Egg shape index (%)	79	71	100	2.9	3.7
Albumen height (mm)	10	7.0	14	1.5	14
Albumen width (mm)	64	55	82	3.7	5.8
Albumen weight (g)	37	31	46	3.0	8.1
Albumen index (%)	16	11	26	2.7	17
Albumen percentage (%)	61	55	76	2.3	3.8
Haugh unit	100	83	114	6.4	6.4
Yolk weight (g)	17	15	19	0.7	4.0
Yolk width (mm)	37	34	42	1.3	3.6
Yolk height (mm)	14	10	18	1.2	8.5
Yolk percentage (%)	23	18	52	2.5	11
Yolk index (%)	47	42	49	1.5	3.4
Albumen:yolk ratio	2.6	2.1	3.7	0.3	10
Yolk colour	11	11	13	0.5	4.3
Shell weight (g)	6.1	3.7	7.6	0.6	10
Shell thickness (mm)	0.4	0.3	0.4	0.02	4.4
Shell percentage (%)	10	6.7	12	0.8	8.6
Shell breaking strength (g)	4852	3129	6952	731	15

CV: coefficient of variation; Min: minimum; Max: maximum; SEM: standard error mean; egg shape index = egg width/egg length $\times$ 100; albumen percentage = albumen weight/egg weight $\times$ 100; albumen index = albumen height/albumen width $\times$ 100; yolk percentage = yolk weight/egg weight $\times$ 100; yolk index = yolk height/yolk width $\times$ 100; albumen:yolk ratio = albumen weight/yolk weight; shell percentage = shell weight/egg weight $\times$ 100.

The changes in egg quality indices of individually caged ISA Brown hens between 25 and 30 weeks of age are presented in Table 2.6. The height of the albumen decreased from 11 mm at 25 weeks of age to 9.3 mm by 30 weeks ($p < 0.001$), while the width of the albumen increased from 64 mm in the 26th week to 66 mm in the 30th week ($p < 0.001$).

Table 2.6 - Changes in egg quality indices of individually caged ISA Brown hens between 25 and 30 weeks of age (n = 264).

Parameters	Age of Hen (Weeks)						SEM	p-Value
	25	26	27	28	29	30		
Egg height	55.09 ^a	55.15 ^a	55.3 ^{ab}	55.06 ^a	55.7 ^{ab}	55.9 ^b	0.25	0.068
Egg width	43.77	44.0	43.7	43.9	43.9	43.8	0.19	0.914
Shape index	79.5 ^a	79.83 ^a	79.19 ^{ab}	79.80 ^{ab}	79 ^a	78.4 ^b	0.42	0.182
Albumen height (mm)	10.9 ^{ab}	11 ^a	10.5 ^{abc}	10.4 ^{bc}	10 ^{cd}	9.3 ^d	0.21	<0.001
Albumen weight	37	36.9	36.9	37.4	37.1	37.6	0.45	0.863
Albumen width (mm)	63.7 ^a	62.2 ^c	64.2 ^a	64.4 ^a	64.9 ^{ab}	66.1 ^b	0.53	<0.001
Albumen index	17.2 ^a	17.8 ^a	16.4 ^{ab}	16.2 ^{ab}	17.1 ^a	14.2 ^b	0.78	0.031
Haugh unit	103.2 ^a	103.3 ^{ab}	100.9 ^{abc}	100.5 ^{bc}	98.6 ^c	96.1 ^d	0.88	<0.001
Albumen:yolk ratio	2.8 ^a	2.8 ^a	2.7 ^b	2.6 ^b	2.5 ^b	2.6 ^b	0.03	<0.001
Yolk weight	16.8 ^a	17.4	17.1 ^a	17 ^a	17 ^a	16.9 ^a	0.1	0.009
Yolk height (g)	13.1 ^a	13.2 ^a	13.9 ^b	14.3 ^{bc}	14.6 ^c	14.2 ^{bc}	0.16	<0.001
Yolk width	36.1 ^a	35.9 ^a	36.7 ^b	37.3 ^c	37.2 ^{bc}	37.2 ^c	0.17	<0.001
Yolk index	46.8 ^a	48.6	46.4 ^{ab}	45.6 ^b	45.7 ^b	45.5 ^b	0.35	<0.001
Yolk colour	11.3	11.5 ^a	11.6 ^a	11.9	11.7 ^a	11.6 ^a	0.06	<0.001
Shell weight	6	6.2 ^a	6.3 ^a	6.3 ^a	5.4	6.4 ^a	0.08	<0.001
Shell breaking strength (g)	4886 ^a	5043 ^a	4839 ^a	4806 ^{ab}	5004 ^a	4533 ^a	107.1	0.015
Shell thickness (mm)	0.40 ^{ab}	0.40 ^a	0.41 ^{abc}	0.42 ^{bc}	0.42 ^{abc}	0.42 ^c	0.002	0.058

abc Means within columns with different superscript are significantly different at the 5% level of significance. SEM – Standard error of mean

Hen performance and egg quality traits

Correlations showing the relationships between hen performance characteristics and egg quality traits of the experimental hens are presented in Table 2.7. Briefly, yolk colour was weakly associated with ADFI ($r = 0.36$, $p = 0.02$) and FCR ($r = 0.38$, $p = 0.02$). FCR had a negative association with albumen weight ($r = -0.49$, $p = 0.006$). There was no association between ADFI and albumen height ($r = 0.18$, $p = 0.21$), IBW and albumen height ($r = -0.04$, $p = 0.79$), or FBW and albumen height ($r = -0.06$, $p = 0.69$).

Table 2.7 - - Correlation (r) between hen performance and egg quality parameters of individually caged ISA Brown hens aged between 25 and 30 weeks old (n = 44).

	Egg Shape Index	Albumen Height	Albumen Weight	Yolk Weight	Yolk Height	Yolk Color	Albumen:Yolk	Shell Weight	Shell Thickness	Eggshell Breaking Strength
Egg produced/ hen/ day	-0.12	0.08	-0.13	0.17	0.04	0.04	-0.12	-0.16	0.06	0.001
Egg weight	0.08	0.31*	0.91***	0.33*	0.38**	-0.11	0.54**	0.73***	-0.13	-0.004
Egg mass	-0.01	0.32*	0.69***	0.44**	0.33*	-0.08	0.39**	0.51**	-0.07	-0.003
Feed intake	-0.03	0.18	-0.04	0.15	0.23	0.36*	-0.18	0.08	0.09	0.03
Initial body weight	-0.07	-0.04	0.09	-0.12	0.14	0.11	-0.001	-0.08	-0.005	-0.07
Final body weight	0.01	-0.06	0.03	0.03	0.15	0.20	-0.05	0.001	0.10	0.007
Body weight change	0.09	-0.05	-0.05	0.19	0.09	0.23	-0.08	-0.09	0.17	0.09
Feed conversion ratio	-0.05	-0.03	-0.49**	-0.08	0.01	0.38*	-0.43*	-0.28	0.12	0.02

*** coefficients with a *p* value < 0.0001; ** coefficients with a *p* value < 0.01; * coefficients with a *p* value of ≤ 0.05.

2.5 Discussion

The monitoring of hen and egg traits in commercial layer flocks is a critical practice to ensure high levels of productivity and to inform on management intervention when necessary. While simple statistics can be collected to assess the extent of variation in flock body weight and egg quality variables, it is not possible to obtain such data on other variables. According to the ISA Brown breed standard (ISA Brown, 2017), hens aged between 25 and 30 weeks are capable of having an average EP of 95.6%. The average of 98% recorded in this study and specifically, 82% of the flock laying above the breed standard confirms that the hens surpassed the expectation of the breed standard, agreeing with the findings of Parkinson et al (2015). The average EP of the hens was 98% at week 25 and hovered around this rate through most of the experimental measuring period. This shows that once peak production is attained, egg production is maintained at least until 30 weeks of age. Hence, this study shows that EP was not an important source of variation in the hen flock. Following the experimental period, nine of the birds were excluded due to mortality (1.5%) or not being in lay (0.4%). The weight of the hen egg is an important gauge of preference, quality and profit in the layer industry (Khan et al., 2004). The overall average egg weight of 61 g recorded in this study was consistent with the ISA Brown suggested target of 61.5 g for hens between 25 and 30 weeks old and with an average CV of 6%, suggests that in an early-lay flock, egg weight does not vary considerably. This is corroborated by several studies which reported no significant difference in egg weights from ISA Brown hens (Rizzi et al., 2006; Zemková et al., 2007). However, egg weight increased significantly from week 25 to week 30, at 60 g and 63 g, and this increase may be due to an increase in hen age, as similarly reported by Lacin et al (2008). The egg weight recorded at 30 weeks in this study was similar to the results of Lee et al (2017), who reported an average egg weight of $64.1\text{g} \pm 3.6$ for the HyLine Brown breed of hens at 30 weeks of age. Conversely, the average eggs from the current study were heavier, 61 g, when compared to the

work of Roberts et al (2013), who recorded an average egg weight of 57.6 g in their study with ISA Brown hens aged between 25 and 40 weeks old. Collectively, EP and EW were relatively stable over the course of the current experimental period, suggesting that these traits were not an important source of variation in the study.

An ADFI of 114 g per hen per day offered on an *ad libitum* basis is recommended by the breed standard for hens within 25 to 30 weeks (ISA Brown, 2017). The average ADFI for the total experimental period, 124 g, was 9% higher than the breed target. The wide range in ADFI, spanning 115 g in the lower quartile of the flock to 169 g in the upper quartile of the flock, together with the 12% CV in this study, shows dissimilarities in consumption among individual birds and indicates the differences in individual hen appetite and nutritional requirement for performance and maintenance. An average FCR of 1.93 is suggested by the ISA Brown breed standard for hens between 25 and 30 weeks. However, the mean FCR recorded in this study was 2.10, and this may have been influenced primarily by the high feed intake of the birds in the present study. The 12% average CV shows that considerable differences and variation exist in feed conversion ratio across the flock. There was no effect of time in the FCR between the 25th and 26th week of age, however, weekly FCR was seen to improve from 2.17 in the 25th week to 2.13 on the 30th week of age.

A total of 88% of the birds in the flock weighed more than the suggested body weight (1660 g) at 21 weeks and 83% weight more than the 1835 g expectation at 30 weeks. This increased BW was similar to observations from Parkinson et al (2007) and Parkinson, Roberts and Horn (2015), who reported that heavier animals have a greater energy and nutrient requirement for maintenance which will increase feed intake, worsen FCR, and subsequently may affect general health of the hens later in lay. The overall CV for initial and final body weight, which may be used as a measure of uniformity, increased from 7% at 21 weeks to 9% at 30 weeks. This suggests that body weight, although above breed standard, is relatively stable in the early

stages of lay in hens offered *ad libitum* access to feed. The body weight change between initial and final body weight varied markedly in the current study and this might have implications for greater variation in body weight later in mid- and late lay.

The positive association between FCR and BW change in the present study is consistent with a study by Lacin et al (2008), who reported positive associations between BW and FCR. Similarly, Akter et al (2018) reported that hens classed as highly feed efficient, with an FCR value of < 1.8 , had a significantly lighter BW when compared to hens ranked as poorly feed efficient, $FCR > 2.1$. The positive association between hen BW and ADFI in the current study was expected and in agreement with Lacin et al (2008). The weak associations observed between ADFI and EW and between FBW and EW suggest that increased feed intake and BW had a minor role in the early stages of lay in influencing the weight of the egg. However, as the average BW of hens in this study was in excess of the breed standard, this observation may not apply in flocks with a greater range of BW or a lower average BW. Hence, in the context of this study, the lack of association between BW parameters and EP, and ADFI and EP, suggests that bigger hens did not lay more eggs, and neither did increase feed consumption contribute to more eggs being laid. Various researchers (Siegel, 1961; Festing & Nordskog, 1967; Poggenpoel & Duckitt, 1988) have suggested that it may be more economical to have lower weight, early-maturing hens which lay consistently compared to heavy hens which lay extra-large eggs. Nordskog and Briggs (1968) also investigated the relationship between BW, EW, and EP, and suggested that finding a middle ground to control feed intake and hen body weight while maintaining commercially acceptable egg weights is necessary to maintain uniformity and optimize production resources. Parkinson, Roberts and Horn (2015) also highlighted the importance of controlling body weight distribution within the breed standard to reduce variation in performance traits among flocks. It may be suggested that, although hen BW plays a significant role in attaining sexual maturity and EP, it may not be as critically important after

the maximum rate of ovulation has been reached at about 24 weeks. As seen in the present study, there was no association between EW and BWC.

Albumen height is an important measure of egg quality and tends to be greatest in early lay and then declines gradually with age (Perić et al., 2017). In the present study, the wide range of 7 mm to 14.2 mm and the average CV of 14% for albumen height reflect differences in individual albumen quality of eggs in the early stages of lay. There was a time effect on albumen height, which was higher in the 25th and 26th week, compared to the 29th and 30th week. The average albumen height obtained in the present study was similar to that reported by Lee et al (2017). However, it was higher than the values obtained from the study by Roberts et al (2013) on eggs from a commercial caged layer flock, within 25 to 40 weeks of age. Albumen height can also be corrected for egg weight to determine the Haugh unit (Haugh, 1937), which has been adopted by the poultry industry as a standard measure of albumen quality. In the present study, the Haugh unit (HU) of all eggs assessed ranged from 83 to 114, with an average CV of 6.4%, suggesting a generally low level of variability for this egg quality measurement in the flock. There was a significant time effect on the weekly HU with a reduction from 103 in the 25th week to 96 in the 30th week. This decline in HU may be associated with the increase in age of the hens also reflected in the decrease in albumen height across the laying period. However, it was surprising to see a time-related decline over such a relatively short experimental period. The average yolk index recorded in this study was 47%, ranging from 42% to 49%. A higher yolk index implies an increase in quality (Fayeye et al., 2005), as the curved shape of the yolk flattens out as the egg ages (Kelly, 2003). The CV of 3% for yolk index from this study suggests uniformity in yolk quality in early-laying hens between 25 and 30 weeks of age. The average yolk index in the current study was higher than that recorded by Mohammadi et al (2016). Yolk colour is an important visual measure of egg quality which is linked to consumer preference (Dvorák et al., 2007). It is influenced primarily by the content and the profile of pigmentation

carotenoids present in the feed ingredients (Leeson & Summers, 1997; Silva, Albino and Godói, 2005). The average yolk colour score obtained in the current study was similar to that reported in the study by Roberts, Chousalkar and Samiullah (2013) on hens between 25 and 40 weeks of age. The weak association observed for yolk colour and feed intake was expected as yolk colour pigment is primarily influenced by dietary fat-soluble xanthophylls.

Eggshell characteristics are important measures of quality, determining hatchability, suitability for table, and preference by the consumers (Hunton, 2005; Ketta and Tůmová, 2018). The average CVs observed for shell weight and shell thickness suggest uniformity in shell quality across the experimental period. These were similar to those observed in the control group of the study by Mohammadi et al (2016), as well as in the study of early-laying hens reported by Roberts, Chousalkar and Samiullah (2013). Shell thickness remained relatively consistent throughout the experimental period with a slight and significant increase of 0.02 mm between the 25th and 30th week of age. There were no associations between hens ADFI, EW, EP, IBW, FBW, and shell thickness and shell breaking strength. This could be linked to the young age of the hens, and it will be useful to see how these relationships continue or change in the later stages of lay.

In a previous study, it was observed that hens ranked as having a high FCR had greater BW and produced eggs with a lower Haugh unit (Akter et al., 2018). In the present study, the absence of an association between albumen height versus feed intake and BW indices suggests that neither increased feed consumption nor increased hen body weight was associated with albumen quality in early-laying hens. However, as the CV of body weight tended to increase over time (7% at 21 weeks and 9% at 30 weeks) and with the huge variation in BWC, it may be expected that the associations between BW indices and albumen quality could become significant as the hens enter mid-lay, with heavier, inefficient hens producing eggs with lower

albumen quality. It would be of interest to investigate the persistence or changes in these associations in the mid- and late lay stages on an individual hen basis.

2.6 Conclusion

This study reports on the extent of variation in EP, EW, ADFI, BW, albumen, yolk, and shell qualities in a flock of individually caged ISA Brown hens in early stages of lay. The body weight of hens in this study was more than BW guidelines of ISA Brown. Albumen height and ADFI varied considerably between individual early laying birds. Hen body weight and feed consumption at the early stages of lay had only a weak association with egg weight, and there were no significant associations between ADFI and albumen height or between body weight and albumen height. The findings from this study agrees with the hypothesis as it revealed that feed intake, body weight and albumen height of hens varied from as early as 25 weeks old, and these variations are consistent up to 30 weeks of age. Further, increasing feed consumption did not increase egg production nor albumen quality. It can be concluded that there is important variation among hen performance and egg quality traits, as well as associations between early-laying hens and eggs. Further investigation on the persistence and changes in the variation and its association with egg quality is merited to better understand the links between production traits and egg quality and safety.

CHAPTER THREE

3. Hens that exhibit poorer feed efficiency produce eggs with lower albumen quality and are prone to being overweight.

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This is the second result chapter which evaluated the effect of varying feed efficiencies on body weight changes, egg production, nutrient digestibility, and egg quality characteristics of individual ISA Brown hens in the mid laying stage of lay. The hypothesis of this study was that hens which demonstrated a higher feed efficiency had a lighter body weight and improved egg quality.

3.1 Abstract

The hen is an efficient producer of eggs, which is impressively an economic source of high-quality nutrients. Feed efficiency (FE) is an important measure of productivity in the layer industry; however, little is known about how FE differs between individual hens during the egg laying cycle, and the implications for egg quality parameters. Individual 25-week-old ISA Brown hens were observed for 42 days, ranked into three FE groups: High (HFE), Medium (MFE) and Low (LFE) and monitored later in the laying cycle (35 to 40 weeks; $n = 48$ per FE group). The FE groups exhibited different feed to egg conversion ratios ($p < 0.001$) from 35 to 40 weeks. Average daily feed intake and bodyweight was highest ($p < 0.001$) in the LFE group compared to the MFE and HFE group while albumen height, Haugh unit, and amino acid concentrations of the albumen were significantly higher in the HFE groups compared to the LFE cohort ($p < 0.001$). There was no difference in shell characteristics between the three FE groups. This study concludes that FE status established in early lay is a stable variable until at least 40 weeks of age, and overweight mid-laying hens had a poorer albumen quality. The distinct traits of the highly efficient hens may provide important information to poultry breeders and egg producers, towards improving the economics of hen and egg production and generally improve management decision making which are usually made based on accepting the expected average performance of a cohort of animals.

Keywords: Egg quality, laying hens, performance traits, feed efficiency, amino acids

3.2 Introduction

The feed to egg conversion ratio (FCR) is an important marker of feed efficiency (FE) in the egg industry, as it captures the efficiency with which the quantity of feed consumed by laying hens is converted to egg mass. First described in 1941, the FCR is influenced by genetics (Hess et al., 1941; Miller & Quisenberry, 1958; Reyer et al., 2015), physiological, environmental and management factors (Aviagen, 2011; Agostini et al., 2014), and has improved by 42% from 3.44 in the 1960s to an average of 1.98 in 2010 (Pelletier et al., 2014). An improved (lower) FCR means less feed is required to produce eggs, thus making it a trait which influences breed selection, overall profitability (Bezerra et al., 2013) and a reduced carbon footprint on the environment (Nkrumah et al., 2006; Basarab et al., 2013; Xin & Liu, 2017). Despite its improvements and importance, the inherent variation in FCR between individual hens has received less attention due to the challenges in profiling feed intake, egg production and FCR between individual hens within a flock. Studies have shown that variation in the FE between individual hens is substantial (Lacin et al., 2008; Akter et al., 2018; Anene et al., 2020), however, the influence of this variation on other hen performance characteristics and egg quality traits is not well understood.

Previous work investigating different body weight groups on performance and egg quality showed that light-weight hens between 1400 g to 1500 g on a flock basis consumed less feed, produced lighter eggs, had a lower (better) FCR and a higher Haugh unit score, compared to heavier weight birds (Lacin et al., 2008). The limitation with assessing production variables on a flock basis are that important variation among individuals is not captured and the subsequent inconsistency in voluntary feed intake and hen body weight can result in a poor uniformity in egg production as well as external and internal egg quality characteristics (Parkinson et al., 2015). In addition, the absence of information about individual hens negatively influences nutrition and management decisions, which are usually made, by necessity, based on the

expected average performance of a cohort of animals. A few studies on the FE of individual hens (Miller & Quisenberry, 1958; Wing, 1981), reported that laying hens which were classified as efficient feed converters, consumed less feed per gram of egg mass and were lighter than intermediate and inefficient hens. These studies were however focused on white-shelled hens, and there is no information about how early in the laying phase differences in FCR exist between individual hens and whether those differences may remain at later periods in lay.

The quality of the egg is evaluated by several parameters in the albumen, yolk, and shell, however, the consequences of FCR on measurements of egg quality are relatively unknown. A recent preliminary study showed that in a flock of individually caged hens on the same dietary and environmental conditions, differences in the body weight and feed efficiency of ISA Brown hens aged 55 weeks old were associated with egg quality variables (Akter et al., 2018). That study reported that hens profiled for having poor FCR had a lower Haugh unit, a heavier yolk and a greater concentration of yolk saturated fatty acids when compared with those described as excellent (low) FCR. The limitation of that study was that the investigation was carried out on hens approaching the later stages of the laying cycle, thus there is no information on if or how early in lay, these differences between individual hens are established.

Despite genetic improvements that have produced hybrid hens with high egg productivity, there is a scarcity of scientific knowledge on the onset and persistency of the differences that exist in individual hen FE and its resulting associations with other hen performance traits and egg quality characteristics. Therefore, the objectives of this study were to evaluate the hen performance traits, nutrient digestibility, and egg quality characteristics of individual ISA Brown hens in the mid laying stage of egg production (35 to 40 weeks), ranked based on their retrospective performance during the early lay period (25 to 30 weeks).

3.3 Methodology

Ethics approval

The experiment was conducted at the Poultry Research Foundation (PRF) layer rearing facility, within The University of Sydney Camden Campus, New South Wales, Australia. The procedures and activities conducted in this study, were approved by the University of Sydney Animal Ethics Committee, protocol number AECL 2017/1212 and in accordance with the 8th edition of the Australian code for the care and use of animals for experimental purposes (NHMRC, 2013).

Experimental diet

A wheat soy mash diet containing 16.30% crude protein and 2750 kcal/kg of gross energy was formulated according to the National Research Council (NRC, 1994) to meet the nutritional requirements of the birds as presented in Table 2.1. The same diet was supplied to all experimental birds in individual feeding troughs placed in front of the cages. Fresh diets were measured out weekly and offered, and at the end of the week, feed not consumed was taken out, measured, and recorded.

Onset of lay, egg weight at first lay.

A total of 455 pullets aged 16 weeks, were obtained from a certified grower farm, weighed individually and randomly assigned in 25 × 50 × 50 cm single multitier metal layer cages in the layer research facility. The shed temperature was controlled around 24°C and photoperiod cycles of 16 h light and 8 h darkness were maintained, as recommended by the ISA Brown management guide (ISA Brown, 2017). All hens were watered by automatic nipples installed in the cages and were fed the experimental diet on an *ad libitum* basis. From 16 weeks up to 24 weeks, all hens were monitored for date of first oviposition: the day when the first egg for each individual hen was laid, and the egg laid was weighed and recorded.

Preliminary study, 25 to 30 weeks of age.

When the hens were 24 weeks of age, they were weighed individually, and the weight was recorded as initial body weight (IBW). Data collection started from 25 weeks up to 30 weeks (estimated as the early lay stage), lasting a total of 42 days. During this period, egg output and egg weight were recorded daily, and feed intake measured weekly. The feed (to egg) conversion ratio (FCR) was calculated as the grams of feed consumed per gram of egg mass. All hens were weighed individually at 30 weeks and recorded as final body weight, (FBW). Based on the FCR from the preliminary study period, the experimental hens were ranked into quintiles and the hens in the first, third and fifth quintile were classed into three groups, namely: High feed efficiency (HFE), medium feed efficiency (MFE) and low feed efficiency (LFE). HFE hens had an average FCR of 1.83; MFE, 2.05; LFE, 2.39. The performance and egg quality measurements of the wider cohort of hens from this phase has recently been published (Anene et al., 2020).

Hen performance, 35 to 40 weeks of age.

At 35 weeks of age (estimated as the mid laying stage), a total of 144 hens (n = 48 hens per FE group) from the preliminary study, were randomly assigned to the three FE groups. Each hen was caged individually and distributed randomly throughout the upper, middle, and bottom levels of three-tier layer metal cages. The experiment lasted for 42 days, between 35 to 40 weeks of age. All hens were monitored individually, and the following hen performance parameters were collected: hen IBW was measured at 30 weeks old and FBW at 40 weeks of age using an electronic scale with a digital output. Body weight change was recorded as the difference between initial and final body weight. Egg produced per hen were recorded daily and the egg production (EP) was calculated in percentage as $(\text{total number of eggs produced in seven days} \times 100) / 7$. Feed was distributed once per week and topped-up as required and refused feed was removed and weighed once weekly. Average daily feed intake (ADFI, g) was

calculated as (weekly feed offered – weekly feed unconsumed) / 7. Egg weights (g) were recorded daily per hen using a digital weighing balance, and the average egg mass per hen (g) was calculated as: $EP \times EW/100$; where EP = Egg production, and EW = average egg weight.

External egg abnormality score

All eggs collected were assessed individually for external abnormalities during the egg collection for the preliminary period (25 to 30 weeks) and mid lay stage (35 to 40 weeks). The egg score was the number of eggs with abnormalities that were either double-yoked, dirty, blood stained, shell-less, thin, or soft shelled, cracked, odd-shaped, broken, or frosty. The number of abnormalities in individual hen eggs were totalled, analysed, and compared on an FE basis across the two experimental periods. Egg quality analysis was carried out as described in Chapter 3.

Quantitative amino acid concentration analysis

A total of 20 eggs were randomly collected (n =10 per HFE and LFE group), their albumen separated and based on their dry matter, analysed for concentration of 15 amino acids. The analysis of amino acid contents of the egg albumen was done at the Australian Proteome Analysis Facility (APAF), within Macquarie University, New South Wales, Australia. After first performing acid hydrolysis, the concentrations of amino acids were determined using pre-column derivatisation amino acid analysis with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) followed by separation of the derivatives and quantification by reversed phase high performance liquid chromatography (HPLC) (Cohen, 2001; Cohen & Michaud, 1993). Amino acids were detected by UV absorbance (260 nm). Approximately 150 mg of sample was hydrolysed in 20% HCl for 24 hours at 110°C. Following hydrolysis, an internal standard (DL-Norvaline and α amino butyric acid; AABA) was added to each sample. Following a 1:25 dilution in milliQ water, 10 μ L of the solution was derivatised using an AccQ Tag Ultra Derivatization Kit (Waters Australia). HPLC analysis was based on the method of

Cohen (2001) but adapted for use with an ACQUITY Ultra Performance LC (UPLC; Waters Corp. USA) system. The column employed was an ACQUITY UPLC C18 1.7 μm , 100 mm column (Waters) with detection at 260 nm and a flow rate of 0.7 mL/min. This enabled a 10.2 min. analysis time per sample. Samples were analysed in duplicate, and the results averaged. The use of HCl as the hydrolysis reagent converts the amino acids asparagine and glutamine to their acid forms, aspartic acid, and glutamic acid, respectively. In the presence of HCl, the amino acid tryptophan is destroyed while cysteine is partially destroyed. Therefore, quantitation for cysteine and tryptophan could not be undertaken by this hydrolysis method.

Nutrient digestibility

At 45 weeks of age, 60 hens, $n = 20$ hens per FE group, were randomly selected to be used for a 72-hour nutrient digestibility study. The diet was measured out and fed to the selected birds on an *ad libitum* basis for a total of 8 days, comprising of a 5-day adaptation period to the diet and then a 3-day excreta collection period. Aluminium trays were laid underneath the cages of selected birds to capture total excreta (g) over a period of 72 hours for the determination of apparent metabolizable energy (AME; MJ/kg) and nitrogen retention (N%). At the end of the collection period, total feed intake was calculated, and the total excreta collected was then joined per feed efficiency group, thoroughly homogenised, weighed, and stored in a freezer at -20°C . From the homogeneous mass, known quantities of excreta samples were dried in crucibles in a forced ventilation oven at 70°C for 72 hours to constant weight to determine DM. Excreta were freeze-dried for gross energy and N analysis. Wet excreta dry matter (DM) was calculated by correction for the loss of moisture during both freeze- and oven-drying. Feed samples were also dried and analysed for crude protein (CP), energy efficiency (EE) (AOAC, 2016), and results were expressed on a DM basis. From the results of laboratory analysis information of DM intake, metabolizable coefficients of DM, CP, gross energy (GE), value of

apparent metabolizable energy (AME) and AME corrected for nitrogen retention (AMEn) of the feed and nitrogen retention per day were obtained.

DM digestibility (%) was calculated as: $\frac{\text{Dry matter intake}_{\text{feed}} - \text{Dry matter output}_{\text{excreta}}}{\text{Dry matter intake}_{\text{feed}}} \times 100$

Gross energy was analysed using an adiabatic bomb calorimeter (IKA Werke, C7000, GMBH and CO., Staufen, Germany), and the GE retention was calculated as:

$$\text{GE Retention (MJ/kgDM)} = \frac{\text{Dry matter intake} \times \text{GE}_{\text{diet}} - \text{Dry matter output} \times \text{GE}_{\text{excreta}}}{\text{Dry matter intake} \times \text{GE}_{\text{diet}}}$$

Nitrogen (N) contents of diets and excreta were determined using an elemental analyser (Leco Corporation, St Joseph, MI) and N retention was calculated from the following equation:

$$\text{N Retention (\%)} = \frac{\text{Feed intake} \times N_{\text{diet}} - \text{Excreta output} \times N_{\text{excreta}}}{\text{Feed intake} \times N_{\text{diet}}}$$

The AME values of the diets on a DM basis were calculated from the following equation: The AME was determined using the total collection method of Bourdillon et al., 1990. The AME values were converted to AMEn (AME for zero N retention in body). The AME and AMEn were calculated as:

$$\text{AME} = \frac{\text{DM intake}_{\text{diet}} \times \text{Gross energy}_{\text{diet}} - \text{DM output} \times \text{Corrected GE}_{\text{excreta}}}{\text{DM intake}_{\text{diet}}}$$

$$\text{AMEn} = \text{AME} - 0.036 \times \text{N Retention}$$

Statistical analysis

Hens were ranked based on their FCR performance from 25 to 30 weeks, into quintiles and three FE groups were formed from the lowest FCR (1st quintile), average FCR (3rd quintile) and highest FCR (5th quintile). Data were tested by one-way ANOVA using the PROC GLM procedure of SAS University Edition software (SAS Institute Inc., Cary, NC, USA) with FE

group as the factor. The individual hen within each FE group served as the experimental unit. Differences among least squares means were computed using the pdiff statement in SAS. The effect of FE group on the external egg abnormality score count data were analysed using a Poisson generalised linear model using the GLM function in R (R Core Team, 2020). The emmeans package in R was used to generate the equivalent of least square means $\pm$ standard errors. All results are presented as least square means $\pm$ standard error of the mean (SEM), except the data from the preliminary ranking period. The *p*-value that denotes statistical significance was set as < 0.05 .

3.4 Results

Hen performance

The performance of early-laying hens ($n = 48$ per FE group) between 25 to 30 weeks and mid-laying hens ($n = 48$ per FE group) between 35 to 40 weeks of age from varying FE groups are presented in Table 3.1. The hen groups maintained their FE rankings from the early laying stage up to 40 weeks of age. In the mid laying stage, average daily feed intake (ADFI) was significantly different across the three groups, ($p < 0.001$). The hens in the high feed efficiency (HFE) cohort consumed the least amount of feed, 113 g, followed by the medium feed efficiency (MFE) group, 118 g and the low feed efficiency (LFE) group consumed the most amount of feed daily, 128 g. Final body weight was significantly different across the three FE groups ($p < 0.001$), with hens in the LFE groups weighing the most, followed by the MFE group and HFE hens weighing the least. The egg mass was higher ($p = 0.01$) in the HFE group compared to the LFE group.

Table 3.1 - Average performance variables of individual ISA Brown hens ranked based on FCR during the preliminary ranking period (25–30 weeks) and their subsequent performance in follow up experimental period (35 – 40 weeks), n = 48 hens per FE group.

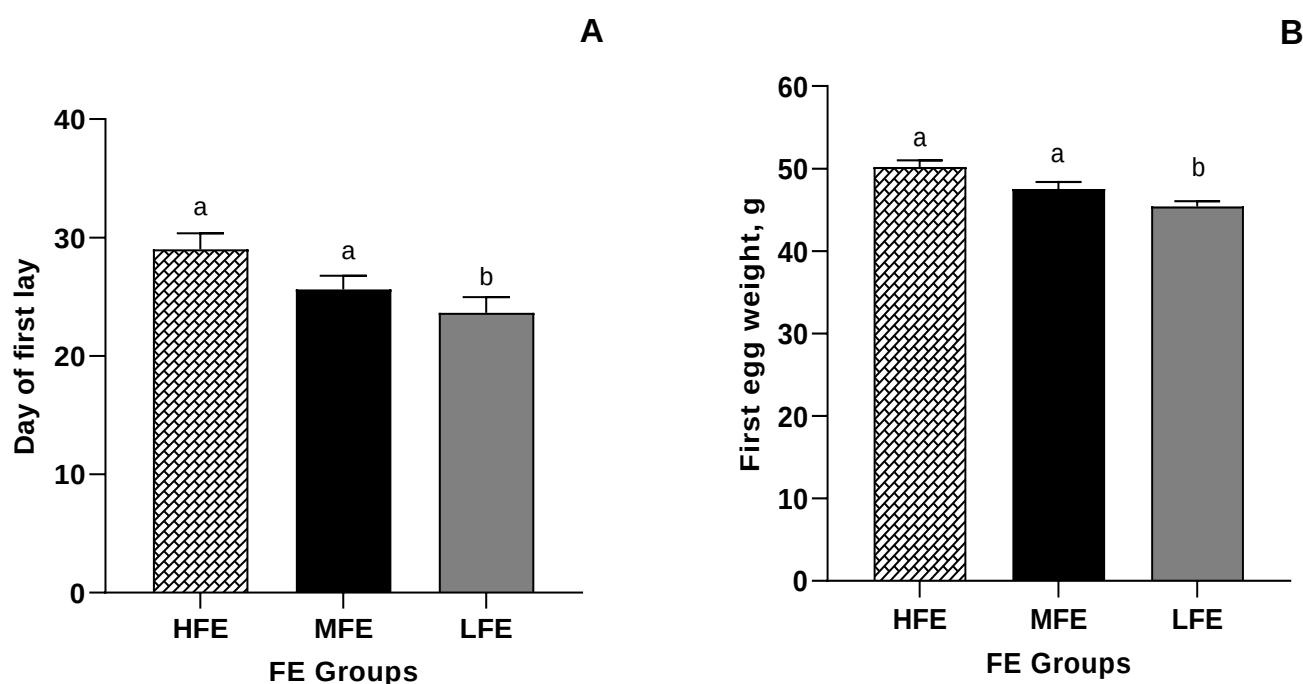
Hen Age	FE	FCR	EP	EW	EM	ADFI	IBW	FBW	BWC
Preliminary ranking period (25 to 30 weeks)	HFE	1.83	98.8	63.5	62.0	113	1760	1923	164
	MFE	2.05	98.8	61.9	60.3	124	1801	2016	215
	LFE	2.39	97.9	60.8	58.3	138	1877	2159	282
Experimental period (35 to 40 weeks)	HFE	1.77 ^a	98.0	64.8	63.6 ^a	113 ^a	1923 ^a	1966 ^a	43.1 ^a
	MFE	1.89 ^b	98.0	63.5	62.2 ^b	118 ^b	2016 ^b	2055 ^b	38.3 ^a
	LFE	2.13 ^c	96.4	62.9	60.6 ^b	128 ^c	2154 ^c	2253 ^c	98.5 ^b
	SEM	0.10	0.60	0.58	0.69	0.35	24	29	13
	P-value	<0.001	0.089	0.06	0.01	<0.001	<0.001	<0.001	0.001

FE: Feed efficiency; HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency. SEM: Standard Error Mean. abc: Means within columns with a different superscript are significantly different at the 5% level of significance. EP: Egg produced per hen per day (%); EW: Egg weight (g); EM: Egg mass (g); ADFI: Average daily feed intake (g); FCR: Feed to egg conversion ratio = ADFI/Egg mass; IBW: Initial Body Weight (g); FBW: Final Body Weight (g); BWC: Body weight change (g); Egg mass = Egg weight × egg production/100 (g).

Onset of lay and egg weight at first lay

Figure 3.1A presents the number of days until first oviposition while Figure 3.1B shows the weight of the first egg laid by individual ISA Brown pullets from between 16 weeks until 24 weeks old, across the three FE groups (n = 48 per FE group). All pullets laid their first egg within a 57 – day window, which is the difference in days between the first oviposition and the last first oviposition of the entire group. The average onset of lay (day first egg was laid) for hens in the HFE cohort was day 29, which was later ($p = 0.01$), than the LFE hens that on

average laid six days earlier, on about day 23. However, the weight of the first egg laid was significantly heavier in hens from the HFE cohort 50.2 g vs 45.4 g $\pm$ 0.82; $p = 0.009$) compared to hens from the LFE cohort.



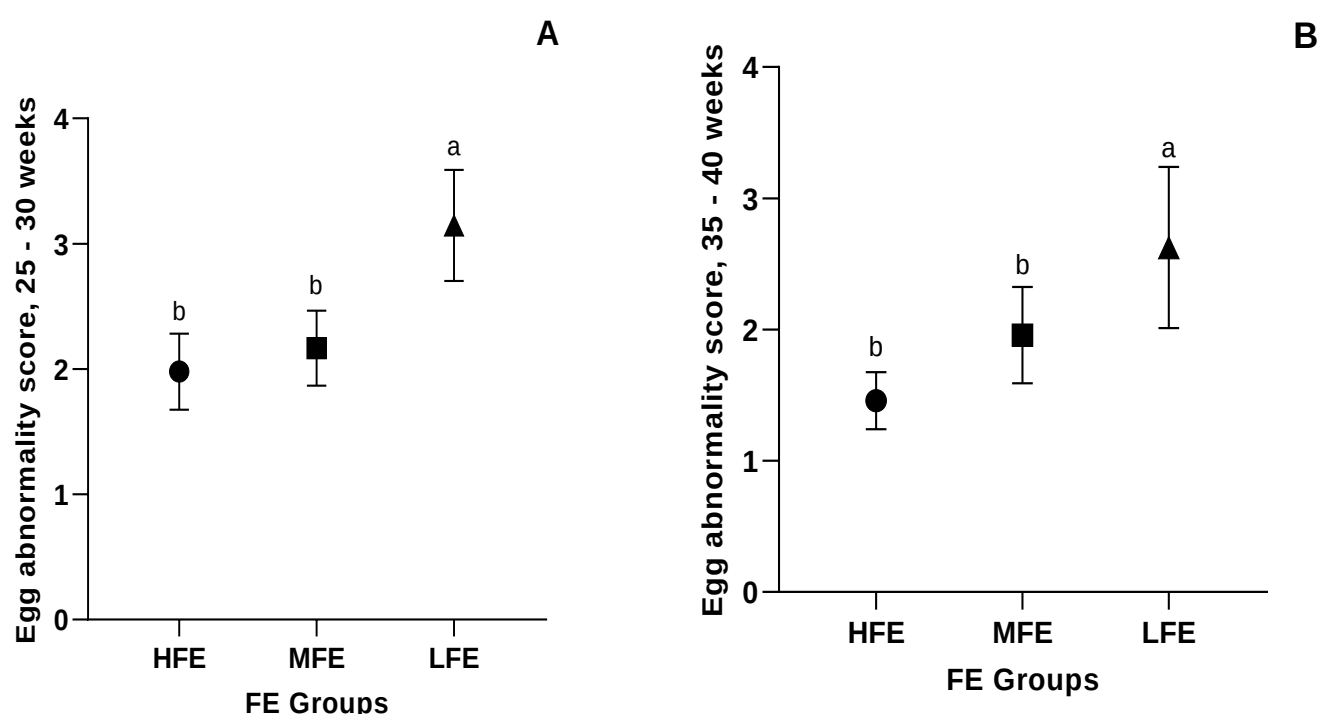
FE: Feed efficiency; HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency. abc Columns not sharing a common superscript are significantly different at the 5% level of significance.

Figure 3.1 - Day of first egg laid across a 57- day onset of lay window from 16 – 24 weeks of age (A) and weight of first egg (B) of individual ISA Brown hens ranked based on FE (n = 48 per FE group).

Egg abnormality score

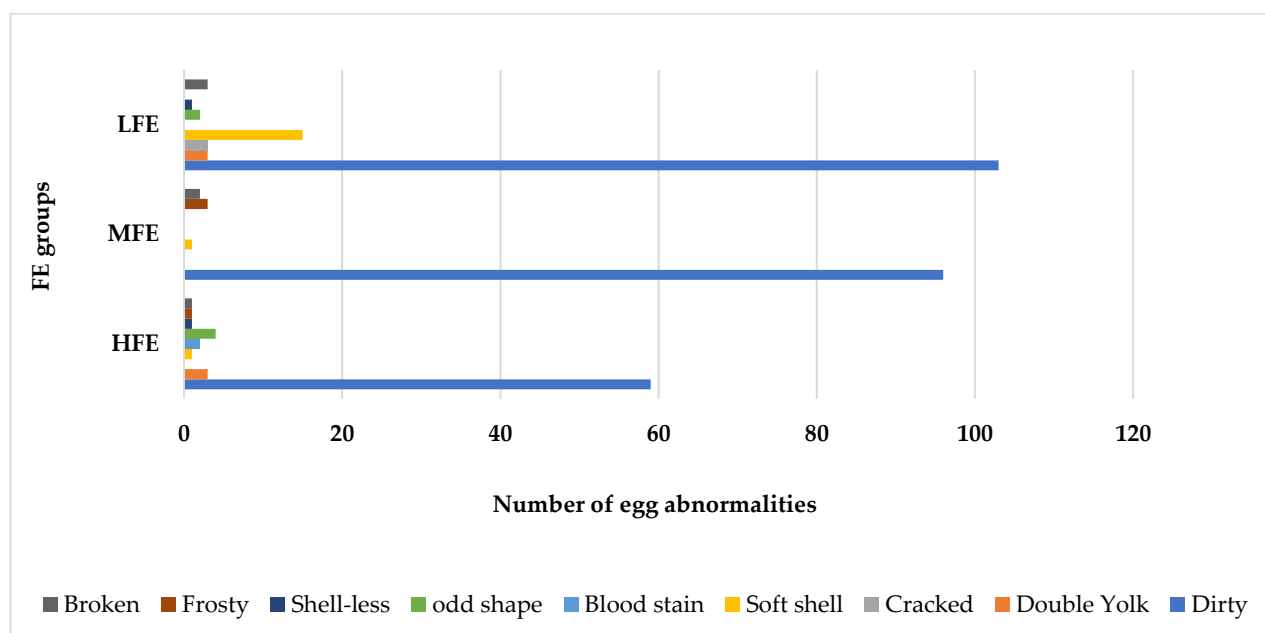
The average egg abnormality score for individual ISA Brown hens in the early laying period (25 to 30 weeks) and the mid laying period (35 to 40 weeks) are presented in Figure 3.2A and 3.2B respectively. Average egg abnormality score was higher ($p < 0.01$) in the LFE cohort compared to the HFE group in both experimental periods. The number of egg abnormalities collected from hens in the mid laying stage (35 to 40 weeks) for the HFE, MFE and LFE groups

are described in Figure 3.3. Briefly, dirty eggs constituted the highest percentage of egg abnormalities observed, and was highest in the LFE cohort, with 103 dirty eggs collected over a 42-day period, followed by the MFE group with 96 dirty eggs and was least in the HFE group, with 59 dirty eggs over the 42-day experimental period. The number of soft-shelled eggs was also highest in the LFE group, as a total of 15 soft shelled eggs were collected from the LFE group across the 42-day experimental period of mid laying hens, compared to one each collected from the MFE and HFE groups.



FE: Feed efficiency; HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency. abc: Means not sharing a common superscript are significantly different at the 5% level of significance

Figure 3.2 - Egg abnormality score of individual ISA Brown hens based on differences in their feed efficiencies, n=48 hens per FE group aged (A) 25 to 30 weeks and (B) 35 to 40 weeks



HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency

Figure 3.3 - The number of abnormalities in eggs from individual ISA Brown hens of varying feed efficiencies in the mid laying stage aged between 35 to 40 weeks (n =48 per high, medium, and low FE groups).

Egg quality

The albumen, yolk, and shell quality characteristics of eggs from hens of different FE groups between 35 to 40 weeks are presented in Table 3.2. Briefly, albumen height, albumen weight and Haugh unit was significantly higher ($p < 0.001$), in the HFE group followed by the MFE and lastly the LFE group. Yolk weight, yolk:albumen ratio and albumen width were however higher ($p < 0.001$) in the LFE group compared to the HFE group. There were no significant differences in the yolk colour, pH of the yolk and albumen, as well as the shell characteristics across the three FE groups.

Table 3.2 - Internal and external egg quality characteristics of eggs from individual ISA Brown hens within 35 to 40 weeks of age, based on differences in feed efficiency.

Egg quality traits	Hen feed efficiency group (n = 15 per FE group)				
	HFE	MFE	LFE	SEM	P-value
Egg weight (g)	64.5	62.6	61.7	0.811	0.06
Egg width (mm)	44.7 ^a	43.9 ^b	43.9 ^b	0.19	0.01
Egg height (mm)	57.2	57.3	56.5	0.36	0.28
Shape index (%)	78.1	76.8	77.7	0.44	0.11
Albumen height (mm)	11.1 ^a	10.2 ^b	8.8 ^c	0.23	<0.001
Albumen width (mm)	66.1 ^b	65.5 ^b	68.1 ^a	0.85	<0.001
Albumen index (%)	16.6 ^a	15.7 ^a	13.0 ^b	0.50	<0.001
Albumen weight (g)	38.8 ^a	38.1 ^b	37.4 ^c	0.20	<0.001
Percentage albumen weight (%)	61.8 ^a	60.5 ^b	59.3 ^c	0.31	<0.001
Haugh unit	103 ^a	99 ^b	93 ^c	0.98	<0.001
Albumen pH	8.0	7.9	8.1	0.03	0.19
Yolk height (mm)	17.7	17.5	17.4	0.10	0.18
Yolk width (mm)	37.6 ^b	38.1 ^b	39.0 ^a	0.17	<0.001
Yolk index (%)	46.7 ^a	46.1 ^a	45.1 ^b	0.32	0.004
Yolk weight (g)	15.0 ^a	15.4 ^a	16.3 ^b	0.17	<0.001
Percentage yolk weight (%)	23.9 ^a	24.5 ^a	25.9 ^b	0.27	<0.001
Yolk pH	5.9	5.9	6.0	0.03	0.53
Yolk:albumen ratio	0.38 ^c	0.40 ^b	0.43 ^a	0.006	<0.001
Yolk colour	11.5	11.3	11.4	0.094	0.42
Shell weight (g)	6.42	6.41	6.26	0.07	0.25
Shell thickness (mm)	0.41	0.41	0.41	0.002	0.49
Percentage shell weight (%)	10.0	10.3	10.2	0.13	0.29
Shell breaking strength (g)	4607	4818	4995	146.91	0.1

HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency; SEM: Standard Error of Mean; abc: Means within columns with different superscript are significantly different at the 5% level of significance. Egg shape index = egg width/ egg length $\times$ 100; Albumen percentage = albumen weight/ egg weight $\times$ 100; Albumen index = Albumen height/ albumen width $\times$ 100; Yolk percentage = Yolk weight/ egg weight $\times$ 100; Yolk index = Yolk height/ yolk width $\times$ 100; Yolk:Albumen ratio = Yolk weight/ Albumen weight; Shell percentage = Shell weight/ egg weight $\times$ 100.

Amino acid composition

The differences in amino acid composition from the HFE and LFE hen groups are shown in Table 3.3. Albumen from the HFE hen group was seen to have a higher concentration of all amino acids investigated, compared to the LFE groups.

Table 3.3 - The amino acids composition of egg albumen collected from individual ISA Brown hens in the mid stages of lay, based on varying feed efficiencies.

Amino Acid, mg/g freeze-dried albumen	FE group (n = 10 per FE group)			
	HFE	LFE	SEM	P-value
Histidine	21.6	20.3	0.16	< 0.001
Serine	60.9	57.9	0.47	0.0003
Arginine	51.2	48.4	0.42	0.0002
Glycine	31.6	29.7	0.27	< 0.001
Aspartic acid	119.4	114.1	0.90	0.0014
Glutamic acid	91.3	86.7	0.85	0.0006
Threonine	40.6	38.1	0.38	0.0002
Alanine	51.7	49.1	0.41	0.0003
Proline	32.3	30.6	0.24	0.0001
Lysine	61.4	57.6	0.70	0.0012
Tyrosine	33.9	31.9	0.34	0.001
Methionine	35.9	34.3	0.25	0.0003
Valine	62.3	59.2	0.48	0.0003
Isoleucine	48.8	46.6	0.35	0.0003
Leucine	77.5	73.8	0.54	0.0001
Phenylalanine	55.2	52.4	0.39	< 0.001

HFE: High feed efficiency; LFE: Low feed efficiency; SEM: Standard Error of Mean

Nutrient digestibility

The differences in nutrient digestibility parameters are presented in Table 3.4. There was no significant difference in the gross energy retention, nitrogen retention, dry matter digestibility and the apparent metabolizable energy of hens from the varying FE groups, however, dry matter intake and output were lower ($p < 0.0001$) in the HFE cohort, compared to the LFE groups.

Table 3.4 - Apparent metabolizable energy (AME), nitrogen retention and digestibility rate of individual ISA Brown hens in the mid stages of lay, based on differences in their FE. (n = 20 per FE group)

Measurements	HFE	MFE	LFE	SEM	p- value
Dry matter intake (g/day)	102.1a	105.5a	117.2b	2.08	< 0.001
Dry matter output (g/day)	23.5a	24.8a	28.6b	0.79	< 0.001
Dry matter retention (%)	76.8	76.5	75.5	0.60	0.329
Nitrogen retention (%)	57.8	57.9	56.3	1.25	0.602
Gross energy retention (%)	79.5	79.0	79.0	0.49	0.490
Apparent metabolizable energy (MJ/kg) of DM	12.1	12.0	12.05	0.07	0.479
Apparent metabolizable energy (MJ/kg) corrected for N	11.9	12.0	11.8	0.06	0.359

HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency; N: Nitrogen; DM: Dry matter. abc Means within columns with different superscript are significantly different at the 5% level of significance. SEM – Standard error of mean

3.5 Discussion

Retrospective ranking of the laying hen cohort based on FCR in early lay revealed that differences in FCR were primarily driven by variation in voluntary feed intake, with the hens ranked as HFE having 22.7% lower voluntary appetite compared with LFE hens. In contrast, rate of lay and egg weight was a less important factor influencing FCR rankings. Mid laying hens (ranked based on FCR from the 25 to 30-week period) maintained their FCR group differences until at least 40 weeks of age. The hens from the LFE and MFE cohort consumed 11% and 4% more feed respectively, compared to the hens in the HFE group. Egg weight and rate of lay was lower in the LFE group but not significantly different across groups during the 35 to 40-week period. Egg mass was higher in the HFE cohort and the FCR of LFE hens was higher (worse) than that of the HFE hens by 12%. Taken together, these findings suggest that feed intake is the major factor responsible for the differences in FE in hens. Further, the present study shows that once hens commence lay, feed intake, rate of lay, egg weight and egg mass are relatively stable, as the differences between groups established in early lay remained divergent until 40 weeks. The initial and final body weights of the hens in the mid laying stage differed across the three groups. The birds in the LFE group were heavier and those in the HFE group were lighter, similar to the findings previously reported by Akter et al., (2018). Consequently, body weight change between 35 and 40 weeks was significantly higher in the LFE group compared to the HFE group. The relationship between feed intake and hen body weight across the three FE groups agrees with the reports from Arthur et al (1999), which concluded that variation in feed intake is associated with variation in hen body weight and together is associated with differences in maintenance requirements. From this study, the average body weight for hens between 30 to 40 weeks surpassed breed expectation range by 84 g (1945 g in the present study vs 1861g according to the ISA Brown guide (ISA Brown, 2017) for hens between 30 and 40 weeks), however, heavier hens did not have an increased

rate of lay nor produce bigger eggs, which is usually a reason for maintaining higher body weights during laying periods by egg producers. The age of the hen at first egg lay, has a significant effect on the weight of the first and subsequent eggs, where hens that begin to lay at an earlier age, tend to have a lower mean egg weight (Tserveni-Gousi, 1993). In the current study, the LFE hens which were heavier birds came into lay six days earlier and laid smaller first eggs, compared to HFE hens, which were lighter in weight, came into lay six days later and produced eggs which were 10% bigger than the early maturing cohort. In hens, ovulation is governed by body lipid /energy reserves and will not commence until a threshold is reached. The LFE hens likely reached this threshold sooner, due to greater bodyweight and therefore produced their first egg earlier than the HFE hens.

Egg abnormalities constitute an inefficiency for the egg industry. The differences in egg abnormalities formed in the early lay stage, between 25 to 30 weeks, continued into the mid lay stage, between 35 to 40 weeks, with eggs from the LFE group having more abnormalities compared to HFE hens. Dirty and soft-shelled eggs accounted for the prominent abnormalities observed, and eggs from the LFE cohort had 42% and 93% more dirty and soft-shelled eggs respectively, compared to the eggs from the HFE group. It is difficult to account for why LFE hens produced more dirty eggs, however, during the nutrient retention study, it was observed that LFE hens produced more faecal matter in line with greater feed consumption and this may be a contributory factor. The implications are that in addition to having poor FE, the hens from the LFE group also produced less saleable eggs.

External egg characteristics are an important measure quality and affect consumer preference and apart from egg width, external egg measurements were unaffected by the FE groupings. The smaller egg width in the LFE hen cohort suggests narrower eggs which are not desirable as they negatively influence egg shape index, egg grading and packaging (Duman et al., 2016). Internal egg measurements were strongly influenced by FE group. The higher albumen height

observed in the HFE group in the study indicates a better albumen quality compared to the MFE group and lastly the LFE group. This is similar to findings from Wang et al (2019) who found that eggs with a higher albumen height had a stronger defence against bacterial invasion. When albumen height was corrected for egg weight, the Haugh unit obtained in the HFE cohort was 9.7% higher than that in the LFE group suggesting that eggs from the HFE hens will potentially provide more protection against environmental shock and microbial invasion to the yolk. Further, it suggests that the albumen from the HFE hens will thin slower and thus have longer storage potential, compared to the eggs from the LFE group of hens (Nys & Guyot, 2011). The higher albumen index obtained in the HFE group, and the higher albumen volume as observed in the HFE hen cohort, coupled with the functions of the chalazae which maintains the yolk in the centre of the egg, suggests a well-rounded coverage of thick albumen around the yolk, ensuring maximum protection of the egg yolk against bacterial penetration and protecting it from physical shock damage and bacterial invasion (Van Tijen, 1972). The bigger yolks observed in the LFE hen cohort in the current study can be linked to a reduction in the strength of the vitelline membrane, and associated with a higher occurrence of yolk rupture (Réhault-Godbert et al., 2019). During storage, this may affect negatively on the egg, predisposing it to bacterial invasion compared to eggs from the HFE group. The higher yolk:albumen ratio, observed in the LFE group when compared to the HFE group indicates that excess lipids not used for egg production or body maintenance were stored up as body fat or deposited in the yolk (Nys & Guyot, 2011), and this may be linked to the higher feed intake and body weight. Further, body fat and lipid synthesis require more energy from the feed compared to albumen production, thus, the differences in appetite and body weight between LFE and HFE hens may also be credited to increased yolk weight of eggs in the LFE group compared to the HFE group. This is supported by the study of Hurnik et al., (1977), who reported a positive correlation between yolk weight and feed consumption. The wider yolk

width observed in the LFE group compared to those from the HFE groups, suggests an increased water content in the LFE yolks, a consequence of liquid from the albumen moving through a more permeable vitelline membrane and ultimately leading to a reduction in yolk quality (Nys & Guyot, 2011). During storage, eggs with wider width and higher water infiltration will lead to faster egg deterioration. Yolk index, a measure of the spherical nature of yolk and an indicator of quality in eggs, was significantly higher in the HFE group compared to the LFE group. A higher yolk index implies an increase in quality (Fayeye et al., 2005), and just like most egg quality parameters, yolk index worsens during storage. Yolk colour is enhanced through consumption of dietary derived pigments such as carotenoids. There was no difference seen in the yolk colour across the three groups of hens. The lack of difference observed in the yolk colour score across the three groups in this study suggests that increased feed intake as recorded in the LFE hen group, did not increase the yolk colour score. The lack of differences observed for all eggshell parameters across the three FE groups in the present study indicates that among individual ISA Brown hybrid hens in the mid stages of lay, an increase in feed intake and body weight did not influence shell quality.

Essential amino acids are an indicator of protein quality (Tessari et al., 2016) and egg proteins from the albumen have been reported to show antioxidant properties of eggs (Nimalaratne & Wu, 2015). The concentrations of all amino acids profiled from the egg albumen in the current study were significantly higher in the HFE groups compared to the LFE groups, and this may suggest a higher quality as antioxidant compounds have been reported to which can play an important role in increasing the quality and the shelf life of products, due to their ability to reduce oxidation (Benedé & Molina, 2020). The higher levels of phenylalanine and tyrosine seen in the eggs from the HFE group and supported by the finding from Nimalaratne et al (2011), bestow a higher antioxidant capacity compared to eggs from the LFE group. The amino acid profile and bioavailability in the diet is an important determinant of the amino acid profile

of the egg. In this study, all hens were feed the same composition of diet; however, the LFE hens consumed more feed and hence dietary amino acids (when compared with the HFE hens). It is therefore counter-intuitive that there would be lower concentrations of amino acids in the albumen of the LFE birds. This study therefore concludes that an increased efficiency of feed nutrients by the hens in the HFE group increases amino acid deposition in the albumen. Studies have also reported that despite adequate nutrient levels and energy density in offered diet, inefficient hens were not efficiently utilising the nutrients and were consuming a greater amount to compensate for the deficiencies (Grobass et al., 1999b; Panda et al., 2012). Individual birds may differ in their ability to digest feed (Pym et al., 1984), due to variation in the size of the gastro-intestinal tract, digesta retention time and digestive enzyme activity, and research has shown that residual feed intake, a feed efficiency measurement, is correlated with nutrient digestibility (Richardson et al., 2004). Although the nutrient retention across the three efficiency groups in the current study was not varied, the dry matter, nitrogen, and gross energy retention of the HFE hens was seen to be numerically higher than the LFE group. The LFE hens with a larger feed intake rate produced more dry matter output compared to the HFE cohort.

3.6 Conclusion

The findings of this study agrees with the hypothesis and showed that feed efficiency status once attained in early lay, is maintained until at least 40 weeks of age. Hens ranked as HFE had a lower appetite and BW, commenced onset of lay six days late and produced bigger first eggs compared to inefficient hens that laid smaller eggs earlier. It has also demonstrated that in hens aged between 35 to 40 weeks, feed consumption is the major driver of differences in feed efficiency, and to a lesser extent the rate of lay and egg mass. Whereas the eggshell quality was comparable between FE groups, egg abnormality was higher among eggs from poorly efficient hens. From this study, nutrient digestibility and absorption was not varied, however, feed efficiency and egg quality are inseparably associated, with HFE hens producing eggs with higher albumen quality.

CHAPTER FOUR

4. Inefficient hens have higher liver fat content increasing susceptibility to fatty liver haemorrhagic syndrome.

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This is the third result chapter, and the aim of this study was to estimate the effect of varying feed efficiency and body weight on organ characteristics and incidences of FLHS in mid laying hens. The hypothesis of this study was that there are important associations between feed conversion ratio and body weight with organ characteristics and incidences of FLHS lesion scores.

4.1 Abstract

Poor feed efficiency (FE) in laying hens impacts body weight (BW) and may reflect suboptimal health. Fatty Liver Haemorrhagic Syndrome (FLHS) is an important metabolic disease, mostly observed in laying hens and affects egg production and hen performance. The aim of this study was to investigate the relationships of FE and BW on organ characteristics, liver pathology and incidences of FLHS of individually housed ISA Brown hens. Based on feed conversion ratio measured from 25 to 30 weeks of age, 35-week-old hens were ranked into three FE groups (high, HFE; Medium, MFE and Low, LFE) and monitored for six weeks. At 45 weeks, 10 birds per group were randomly selected, weighed and euthanized to assess digestive tract variables and FLHS pathology. Hen BW was positively associated with feed intake ($r = 0.63, p = 0.0002$) and feed conversion ratio ($r = 0.44, p < 0.01$). HFE hens have a lower abdominal fat pad ($p < 0.001$) and liver weight ($p < 0.01$) compared to LFE hens. There was a strong positive association between slaughter weight (SW) and abdominal fat pad ($r = 0.92, p < 0.001$) and liver weight ($r = 0.75, p < 0.001$). FLHS lesion score was higher (worse) in the LFE group than in the HFE hen group ($p = 0.03$), was moderately associated with SW ($r = 0.47, p < 0.01$) and abdominal fat pad ($r = 0.48, p < 0.01$) but strongly associated with liver weight ($r = 0.74, p < 0.001$). The HFE hens had lower liver dry matter ($p = 0.02$), and fat ($p = 0.008$) content, and thiobarbituric acid reactive substances (TBARS) values ($p = 0.03$) compared to the LFE hens. Microscopic examination of liver tissue from LFE hens showed swollen hepatocytes with distended single or multiple cytoplasmic vacuoles compared to the HFE hens. The study concludes that hens with poorer FE obtained from early lay, had more abdominal fat pad, larger livers and were more prone to FLHS.

Keywords: FLHS, laying hens, feed efficiency, body weight, abdominal fat

4.2 Introduction

Variation in bodyweight (BW) and poorly controlled feed intake (FI) can lead to poor or inefficient egg production (Leeson et al., 1997, Lacin et al., 2008). Maintaining BW is a vital objective in hen production systems, as hens weighing above the breed recommendation for their age and phase of production have a poorer feed to egg conversion efficiency (Ocak et al., 2004; Lacin et al., 2008; Gordon et al., 2009; Pérez-Bonilla, Jabbour, et al., 2012;), come into sexual maturity earlier (Summers & Leeson, 1983), produce eggs of lower albumen quality (Pérez-Bonilla, Novoa, et al., 2012; Anene et al., 2021) and have a higher susceptibility to fatty liver haemorrhagic syndrome (FLHS) (Couch, 1956; Walzem et al., 1993; Trott et al., 2013). The liver supports nutrient intake and utilization, lipid and protein synthesis for egg yolk production (Denbow, 2000; Nys & Guyot, 2011), calcium absorption for eggshell formation (Keshavaraz & Nakajima, 1993) and conversion of toxins into water-soluble waste products excreted by the kidneys (Akers & Denbow, 2013). Due to the multiple roles and hence importance of this organ, impaired function may lead to metabolic diseases, reduced productivity, and production of eggs with poor quality.

FLHS is a chronic metabolic disease which has gained attention in recent years as an important health issue in the layer industry. In a survey by Shini et al (2019), FLHS was reported to cause 74% of the total mortality of caged layers in Queensland, Australia. Additionally, Harms et al (1972) and Grimes et al (1991) showed that FLHS can be present in live hens and exhibit as excessively high-energy feed intake, increased body weight, reduction in egg production and engorged pale combs. Sudden death may also occur and at necropsy, the syndrome is characterized by excessive accumulation of fat within the abdominal cavity, a pale and fragile liver sometimes void of structural integrity, multifocal haemorrhages (Shini & Bryden, 2009) and large blood clots in the abdominal cavity as a result of liver rupture (Rozenboim et al., 2016). Although no diagnostic criteria have been developed to determine FLHS in live birds,

it is reported to be induced by the persistence of high oestrogen production (Polin & Wolford, 1977; Stake et al., 1981) and may be seen mostly during the peak production period (between 30 to 40 of age). A certain BW threshold must be attained to trigger follicle development and sexual maturation (Chen et al., 2006). At this time, the heightened levels of oestrogen increases the metabolic activity of the liver to form the lipoprotein precursors required for yolk production (Nys & Guyot, 2011). Overproduction of lipoproteins by the liver triggers hyperlipidaemia, which is not only a common element of FLHS, but also causes insulin resistance, lipid peroxidation, and an inflammatory response. This could lead to a loss in liver function and an increased appetite and feed consumption as the hen attempts to maintain egg production, eventually causing obesity and a poor feed to egg conversion efficiency.

Recent research suggests that considerable variation in feed intake, BW and feed efficiency exists between individually-housed hens kept under similar management and dietary conditions (Akter et al., 2018). These variations have been reported to begin early in the laying period (Anene et al., 2020), and are persistent until at least 40 weeks of age (Anene et al., 2021). However, there is scarce literature on how these differences in BW and FE established in early lay influence liver health conditions, other organ characteristics, and the incidences of FLHS during peak lay. Therefore, the aim of this study was to explore the associations of FE and BW with organ characteristics and occurrences of FLHS of ISA Brown hens ranked as high, medium, and low feed efficient from early in the laying period, under caged conditions.

4.3 Materials and methods

This study was conducted at the Poultry Research Foundation, within The University of Sydney, Camden Campus, Australia. All experimental procedures conducted in this study were approved by The University of Sydney Animal Ethics Committee and were in accordance with the Australian code for the care and use of animals for scientific purposes (NHMRC, 2013).

Preluding experimental set up – 25 to 30 weeks.

The experimental animals, housing conditions, diet composition and performance characteristics measurements used for the selection of hens in this study have been reported in Anene et al (2021). Briefly, 450 ISA Brown commercial strain pullets were obtained from a certified grower at 16 weeks of age, housed individually in 25 × 50 × 50 cm cages within a layer house, fed a common diet and kept under the same environmental conditions. The hens were monitored from 25 to 30 weeks of age for differences in FI, BW, and egg weight, to facilitate subsequent ranking of birds based on FE. All hens were fed a wheat and soybean meal-based mash on an *ad libitum* basis, which consisted of 16.3% crude protein, 2,750 kcal / kg metabolizable energy 0.82% total lysine, 0.42% total methionine, 4.0% Calcium and 0.4% available phosphorus to meet the recommendations of the ISA Brown bird from early to mid-lay phase. All hens were offered *ad libitum* water through watering nipples placed in each individual cage. At the end of the 30th week and based on the average feed conversion ratio (FCR) of the preceding six-week period, all 450 hens were ranked into quintiles and the hens in the first, third and fifth quintile were classed into three groups: high feed efficiency (HFE), medium feed efficiency (MFE) and low feed efficiency (LFE). The HFE group had an average FCR of 1.83 ± 0.02 , the MFE group had an average FCR of 2.05 ± 0.02 and the LFE group had an average FCR of 2.39 ± 0.02 . From 35 to 40 weeks, hen performance characteristics including; FI, egg weight, egg production, egg mass, FCR and change in BW were monitored and calculated as reported in Anene et al (2021).

Organ Characteristics

At 45 weeks of age, 30 birds from the FE groups ($n = 10$ per FE group) were randomly selected and euthanised by intravenous injection of sodium pentobarbitone via the wing vein. The livers were examined *in situ* and evidence for FLHS was scored as described by Diaz et al (1999). Briefly, liver score 0 indicates normal liver, liver score 1 indicates up to 10 subcapsular petechial haemorrhages, liver score 2 indicates more than 10 subcapsular petechial haemorrhages, liver score 3 to 5 indicates focally extensive to massive subcapsular haemorrhages. The abdominal fat pad, pancreas, gizzard, and whole intestine were excised and weighed individually using an electronic scale with a digital output. Organ weights were also expressed as a percentage of BW. Tissues from the liver and muscle were snap frozen in liquid nitrogen at collection, then stored at -80°C until assayed. The pH of digesta within the gizzard was determined *in-situ*. Digesta from the distal half of the jejunum and ileum were collected in their entirety and pH measured accordingly. The breast (*Pectoralis major*) muscle was excised and the dry matter content of both liver and muscle were measured according to AOAC procedures (AOAC, 2016). The total fat content of the liver and muscle were determined by the Ankom XT15 method based on AOCS Official Procedure Am 5-04 (AOCS, 2005). Temperature compensated pH values were measured in breast (*Pectoralis major*) muscle at 24 h (ultimate pH) post-mortem as described by Hutchison et al (2014). Muscle and liver colour were determined using triplicate colour measurements for lightness (L^*), redness (a^*) and yellowness (b^*), (CIELAB, $L^* a^* b^*$ system, illuminant D65, 10° standard observer) (Colorimetry International Organisation for Standardisation, 2019). Measurements were taken over the muscle and liver surface at 2.5 cm thick slices using a Minolta Chroma meter (CR-300, Osaka, Japan), after the samples had air-bloomed at 1°C for 1h.

Lipid peroxidation of liver and muscle samples were measured as thiobarbituric acid reactive substances (TBARS) using a commercial kit Cayman TBARS (TCA Method) assay kit (Item

No. 700870) following the instructions of the manufacturer (Cayman, USA) with minor modifications. Briefly, liver and muscle samples were thawed on ice, chopped into small pieces, and washed twice with ice-cold PBS to remove any blood. A total of 25 mg of tissue samples was transferred in to a 2.0 mL safe lock tube containing two 3 mm diameter metal beads. Then, 250 µl of RIPA buffer with protease inhibitor (EDTA; 10 µl/mL) was added per tube and the sample was homogenized using a Qiagen TissueLyser II lysed at a frequency of 30 Hz for 2 minutes. The sample was centrifuged at 16000 x g for 10 minutes at 4°C to remove insoluble materials and the supernatants were then collected for measuring TBARS using Cayman TBARS assay kit.

Liver Histopathology

Liver slices (0.5 to 1.0 cm) collected at post-mortem examination were fixed in 10% neutral buffered formalin and paraffin wax-embedded 4 µm sections were stained with haematoxylin and eosin (H&E) and assessed microscopically at a magnification of X40, by a pathologist unaware of the categorisation based on the FCR. Twelve liver sections were reviewed and the degree of hepatocellular vacuolation characterised by clear, well demarcated, single, or multiple cytoplasmic vacuoles was graded as described by Trott et al (2013). In this grading scheme, sections with no or very rare vacuolation was graded as 0, those with less than 50% of hepatocytes containing vacuoles were as graded 1, liver sections with 50% or greater hepatocyte containing variable size vacuoles or diffuse vacuolation with small vacuoles were graded as 2 while those with diffuse vacuolation with variable sized vacuoles as grade 3. Multiple liver sections from each bird were assessed to determine the grade. Where there was a difference in the grade between sections for a given case, the predominant change in the sections and evidence of haemorrhage were used for grading.

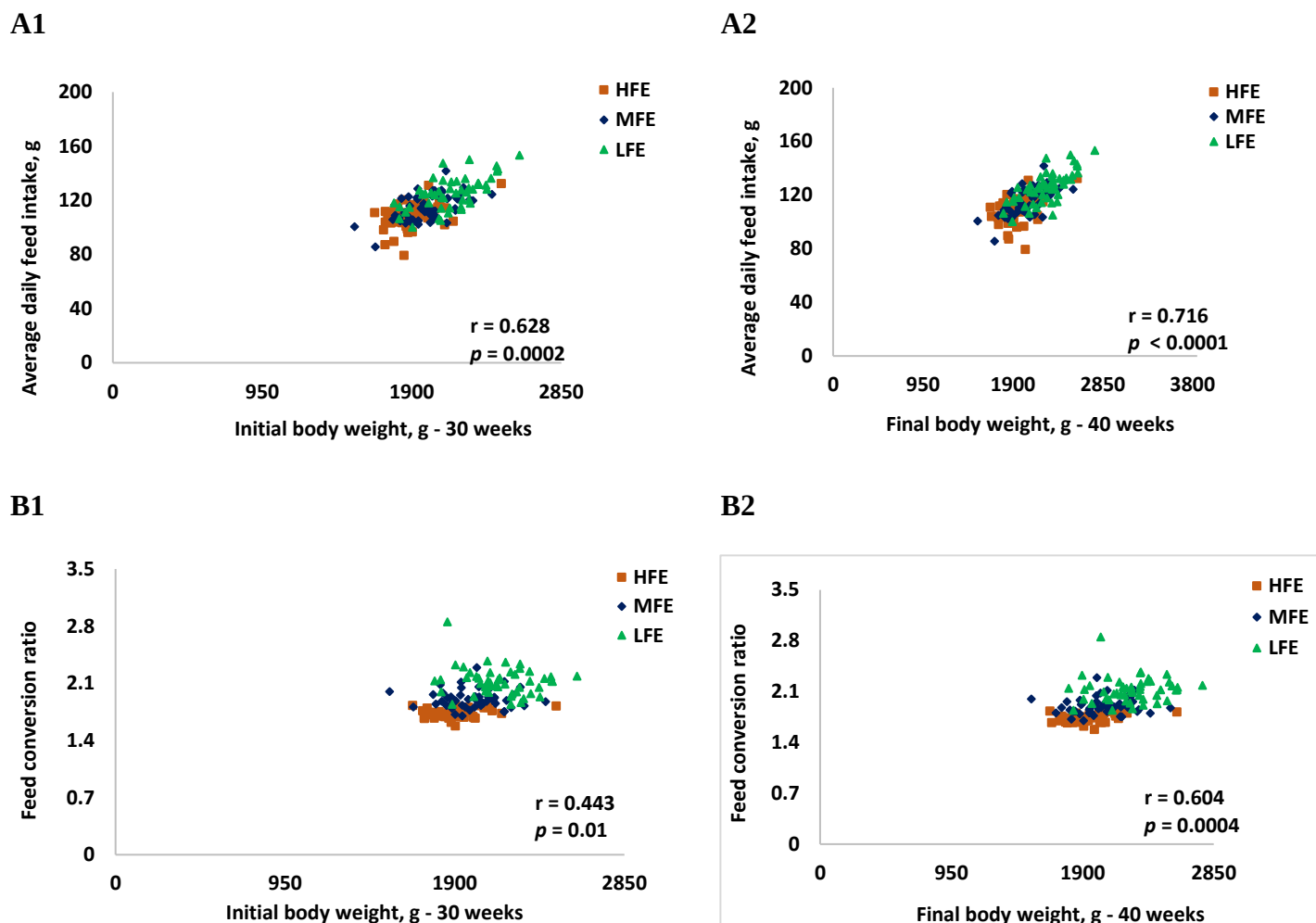
Statistical Analysis

The individually housed laying hen served as the experimental unit. Experimental data were analysed using the generalised linear model procedure of SAS (SAS Institute) with the FE group as the main effect. All data are presented as least square means $\pm$ standard error of the mean (SEM). Differences among least squares means were computed using the pdiff procedure in SAS. Pearson or Spearman correlation coefficients output for production traits and egg quality measurements was generated using the proc corr procedure SAS Institute Inc. The probability value denoting statistical significance was $p < 0.05$.

4.4 Results

Hen performance

The performance traits of individually housed hens between 35 to 40 weeks have been reported (Anene et al., 2021). Summarily, the hen BW at 35 weeks was higher in the LFE birds compared to the HFE hens ($p < 0.001$) and continued to be heavier than the HFE and MFE hens at 40 weeks. The average daily feed intake was higher and the FCR worst in LFE birds, followed by the MFE and least in the HFE ($p < 0.001$). Egg mass was higher in the HFE ($p = 0.01$), but similar in both MFE and LFE. There were no differences in the egg production and egg weight across the three FE groups. Correlation analysis (r) of the hen performance characteristics data from (Anene et al., 2021) are presented in Figure 4.1. Bodyweight at 35 weeks was positively associated with FI ($r = 0.628$; $p = 0.0002$) and this trend was maintained for BW at 40 weeks ($r = 0.716$; $p < 0.001$). The FCR was observed to be positively associated with BW at 35 weeks ($r = 0.443$; $p = 0.01$) and this was maintained at 40 weeks of age ($r = 0.604$; $p = 0.0004$) with heavier birds having a higher (worse) FCR compared to lighter weight hens.



HFE – High feed efficiency; MFE – Medium feed efficiency; LFE – Low feed efficiency; r = coefficient of correlation, p = level of significance.

Figure 4.1 - Correlation of average daily feed intake and initial and final bodyweights (A1 and A2) and feed conversion ratio and initial and final body weights (B1 and B2), of individually housed ISA brown hens aged between 30 to 40 weeks, $n = 150$ (50 per FE group).

Organ characteristics

The digestive organ characteristics of individually housed ISA Brown hens aged 45 weeks old are presented in Table 4.1. At 45 weeks, the LFE hens had a higher BW compared to the MFE hens with the lowest BW seen in the HFE hens ($p < 0.001$). The abdominal fat pad weight was different across the three FE groups with LFE hens having the highest fat pad weight and fat pad weight corrected for BW, followed by MFE and the least in the HFE hen group ($p < 0.001$).

The LFE and MFE hens had bigger liver weights compared to the HFE hens ($p < 0.001$), and this result was consistent when corrected to BW ($p = 0.04$). The total intestinal weight was heavier in the LFE hens followed by MFE hens and least in the HFE hens ($p < 0.001$). However, when corrected for BW, there was no difference across the three FE groups. Although no difference was observed in the weight of the gizzard across the three FE groups, in proportion to BW the gizzard of the HFE hens were seen to be higher compared to the LFE hens ($p = 0.01$). The absolute pancreas weight, pancreas weight as percentage of BW and pH values of the gizzard, jejunum and ileum were not different across FE groups.

Table 4.1 - Organ characteristics of individually housed ISA Brown hens aged 45 weeks old and ranked based of differences in feed efficiency as HFE, MFE and LFE. (n = 30; 10 per FE group).

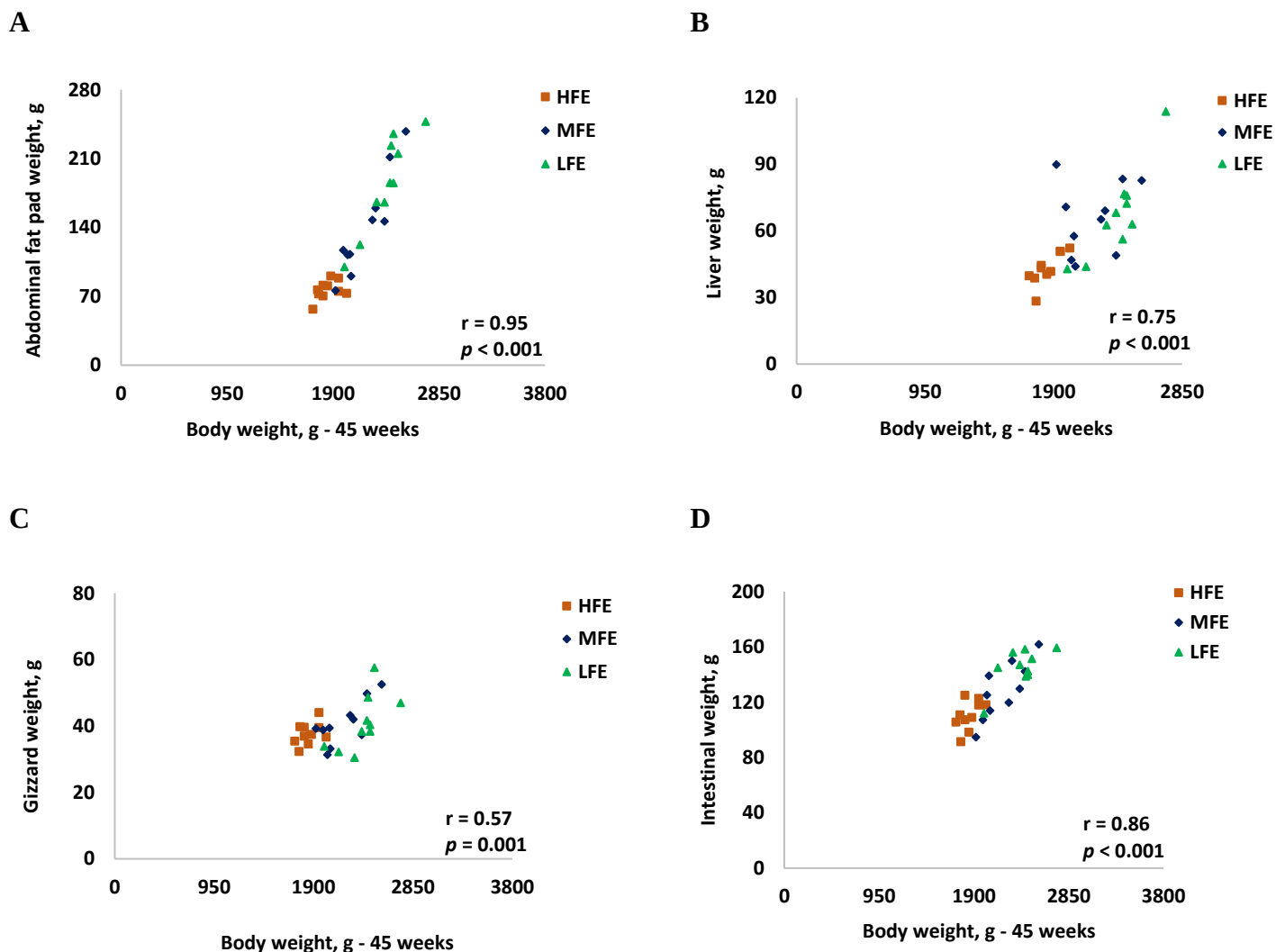
Organ Characteristics	Feed efficiency groups			SEM	p-value
	HFE	MFE	LFE		
BW of bird at 45 weeks of age, g	1852 ^a	2190 ^b	2371 ^c	0.05	< 0.001
Abdominal fat pad weight, g	76 ^a	141 ^b	184 ^c	12.9	< 0.001
Abdominal fat pad weight corrected for BW %	4.1 ^a	6.3 ^b	7.7 ^c	0.43	< 0.001
Liver weight, g	42.9 ^a	65.8 ^b	67.5 ^b	4.91	< 0.001
Liver weight corrected for BW %	2.31 ^a	3.01 ^b	2.81 ^b	0.19	0.04
Intestine weight, g	110 ^a	128 ^b	144 ^c	4.91	< 0.001
Intestine weight corrected for BW %	5.97	5.84	6.12	1.66	0.50
Gizzard weight, g	37.6	40.7	40.8	2.03	0.46
Gizzard weight corrected for BW %	2.03 ^a	1.85 ^{ab}	1.71 ^b	0.07	0.01
Pancreas weight, g	3.19	3.16	3.70	0.26	0.26
Pancreas weight corrected for BW %	0.17	0.15	0.16	0.01	0.25
Ileal pH	7.90	7.90	7.80	0.16	0.88
Jejunum pH	6.20	6.20	6.50	0.19	0.44
Gizzard pH	4.13	4.10	4.18	0.10	0.86

BW: Body weight; HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency; SEM – Standard Error of Mean;

abc - Means within rows not sharing a common suffix are significantly different at the 5% level of probability.

Correlation analysis (r) between hen performance traits and digestive organ characteristics of individually housed ISA Brown hens ($n = 30$) are presented in Figure 4.2 and Table 4.2. There was a strong and positive association between BW and abdominal fat pad weight, ($r = 0.954$;

$p < 0.0001$), liver weight ($r = 0.749$; $p < 0.0001$), gizzard weight ($r = 0.573$; $p = 0.0009$) and intestinal weight ($r = 0.857$; $p < 0.0001$).



HFE – High feed efficiency; MFE – Medium feed efficiency; LFE – Low feed efficiency; r = coefficient of correlation, p = level of significance.

Figure 4.2 - Correlation (r) between body weight at 45 weeks and (A) abdominal fat pad weight (B), liver weight (C) gizzard weight and (D) total intestinal weight of individually housed hens aged 45 weeks ranked based on differences in feed efficiency. ($n = 30$; 10 per FE group)

Hen performance and organ characteristics.

Average daily feed intake had a positive correlation with abdominal fat pad weight ($r = 0.64$, $p = 0.001$), liver weight ($r = 0.55$, $p = 0.002$) and total intestinal weight ($r = 0.76$; $p < 0.001$).

FCR was also seen to be positively associated with the abdominal fat pad weight ($r = 0.55$; $p =$

0.001) and total intestinal weight ($r = 0.54$; $p = 0.001$). No relationship was observed between production traits and the pH of gizzard, jejunum and ileal of the experimental hens.

Table 4.2 - Correlation (r) between performance traits and digestive organ characteristics of individually housed ISA Brown hens aged 45 weeks.

Hen performance traits	Weight (g)						pH		
	Abdominal pad	fat	Liver	Intestine	Gizzard	Pancreas	Gizzard	Jejunum	Ileal
FI	0.64***		0.55**	0.76***	0.13	0.25	-0.06	0.002	-0.14
EW	-0.002		0.16	0.13	0.25	-0.05	0.003	-0.14	0.17
EM	-0.15		0.02	0.03	0.23	-0.19	0.03	-0.16	0.07
FCR	0.56**		0.34	0.54**	0.05	0.15	0.06	0.35	-0.25
EP	-0.26		-0.19	-0.12	0.10	-0.29	0.04	-0.09	-0.09

FI: Feed intake; EW: Egg weight; EM: Egg mass; FCR: Feed conversion ratio; EP: Egg production; *: coefficients with a p value of ≤ 0.05 ;

** - $p < 0.01$; *** - $p < 0.001$

Muscle and liver characteristics

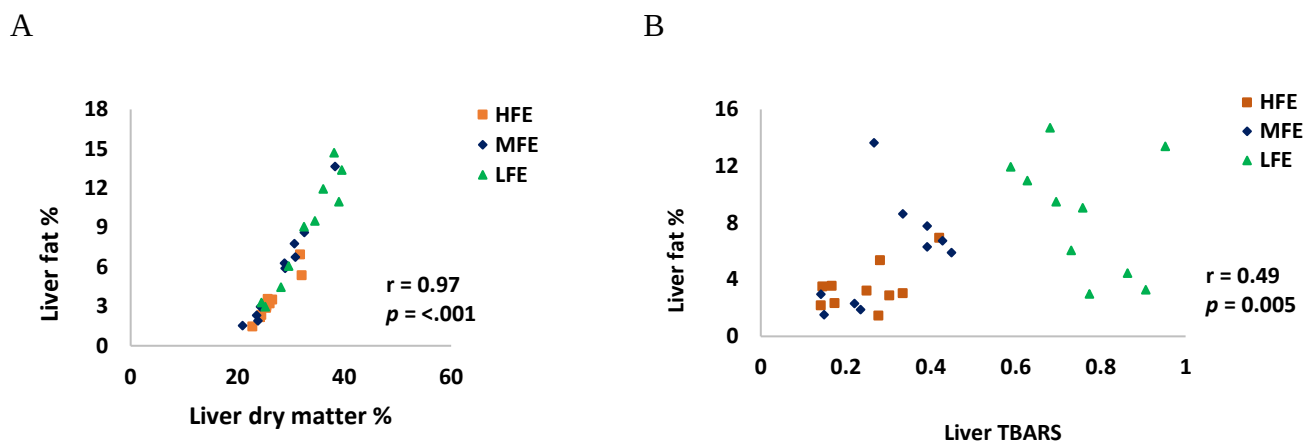
As presented in Table 4.3, the dry matter, total fat composition and colour of the breast muscle (*Pectoralis major*) were not influenced by FE status, however, the liver from the LFE hens were seen to have a higher dry matter percentage ($p = 0.02$) and total fat percentage ($p = 0.02$) compared to the HFE hen group. Liver colour measurements indicated that LFE hens had a higher level of yellowness (b^*) value ($p = 0.004$) while lightness (L^*) and redness (a^*) values were not affected by the differences in FE. As shown in Figure 4.3, a moderate positive correlation was seen between total fat content and TBARS value ($r = 0.49$; $p = 0.005$) while a strong positive association was observed between liver dry matter and liver total fats content ($r = 0.97$; $p < 0.001$), as well as liver b^* value and liver fat percentage ($r = 0.77$; $p < 0.001$).

Table 4.3 - Differences in the muscle and liver composition of individually housed ISA Brown hens aged 45 weeks old, ranked based on feed to egg efficiency (n = 30; 10 hens per group).

Measurements	Feed efficiency			SEM	<i>p</i> -value
	HFE	MFE	LFE		
Muscle					
DM (%)	26.3	26.7	26.4	0.13	0.14
Total fat (%)	0.26	0.41	0.40	0.07	0.34
Lightness (L*)	54.3	54.2	53.7	0.78	0.85
Redness (a*)	1.67	1.93	2.38	0.33	0.31
Yellowness (b*)	4.57	4.24	4.77	0.38	0.60
Liver					
Dry matter (%)	26.5 ^b	28.3 ^b	32.7 ^a	1.50	0.02
Total fat (%)	3.44 ^b	5.77 ^{ab}	8.64 ^a	1.08	0.01
Lightness (L*)	41.2	44.4	47.6	1.93	0.08
Redness (a*)	20.7	21.9	22.1	1.06	0.60
Yellowness (b*)	13.9 ^b	16.4 ^b	20.1 ^a	1.18	0.004

HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency; SEM: Standard Error of Mean; abc Means within rows

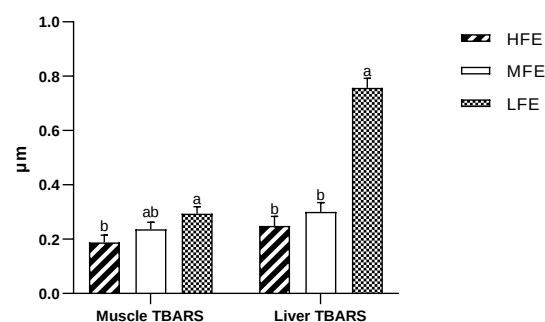
not sharing a common suffix are significantly different at the 5% level of probability.



HFE – High feed efficiency; MFE – Medium feed efficiency; LFE – Low feed efficiency; r = coefficient of correlation, p = level of significance.

Figure 4.3 - Correlations (r) of liver fat with (A) liver dry matter and (B) liver TBARS value of individually housed hens aged 45 weeks ranked based on differences in feed efficiency ($n = 30$; 10 per FE group)

The differences in muscle and liver TBARS based on FE status are shown in Figure 4.4. Hens in the LFE group had a higher lipid peroxidation estimate (TBARS) in their muscle ($p = 0.03$), and liver ($p < 0.001$) tissues compared to both the MFE and LFE hens.



TBARS: Thiobarbituric acid reactive substances High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency; SEM: Standard Error of Mean; abc Means within rows not sharing a common suffix are significantly different at the 5% level of probability.

Figure 4.4 - Effect of feed efficiency on the lipid peroxidation estimated of muscle and liver tissues obtained from individually housed ISA Brown hens aged 45 weeks old ($n = 30$, 10 per FE group).

Fatty liver haemorrhagic syndrome lesion score and liver histopathology

The pictograph of livers obtained from hens of varying FE are presented in Figure 4.5. The livers from the LFE hens were seen to be paler and have more haemorrhages compared to the livers from the HFE.

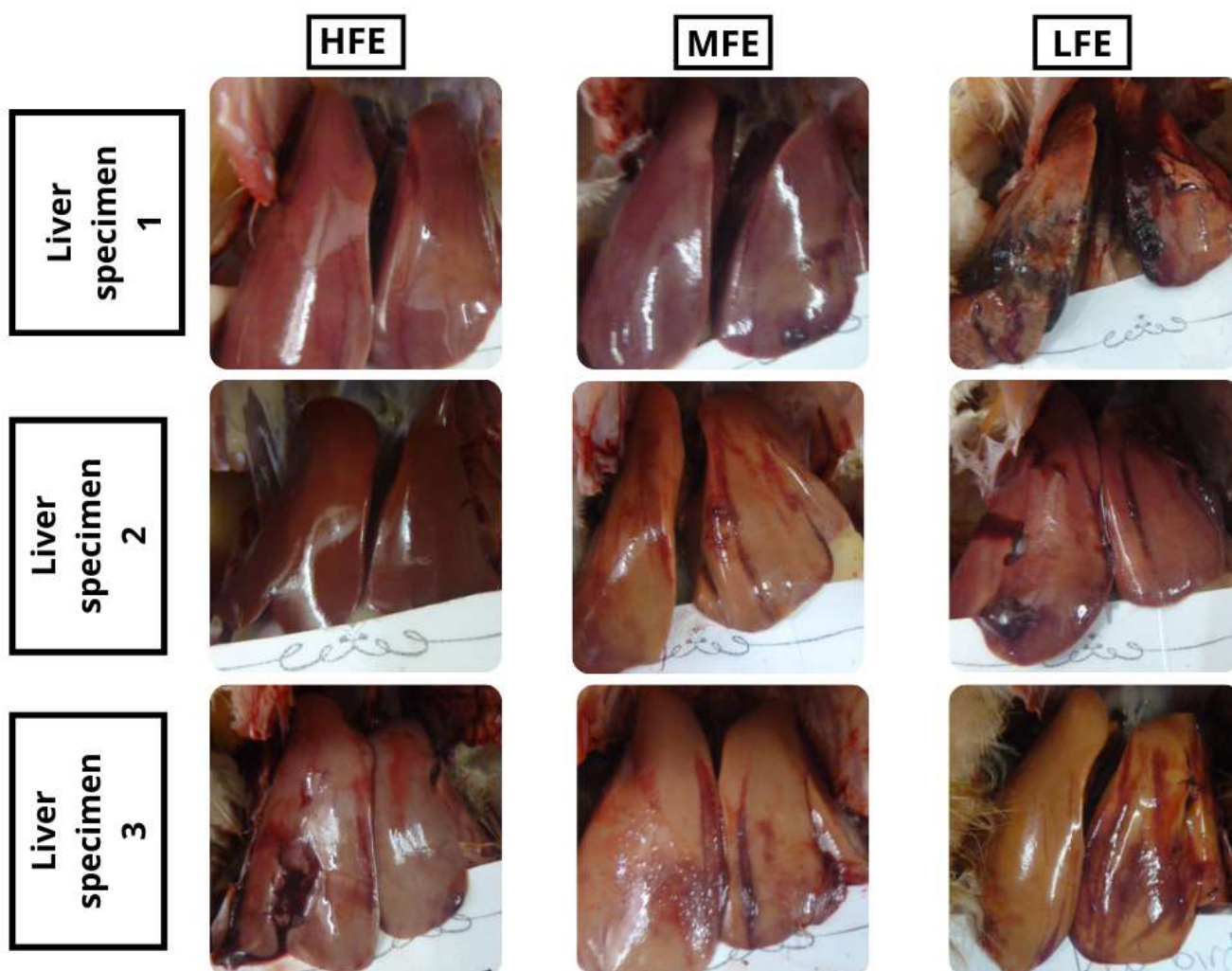
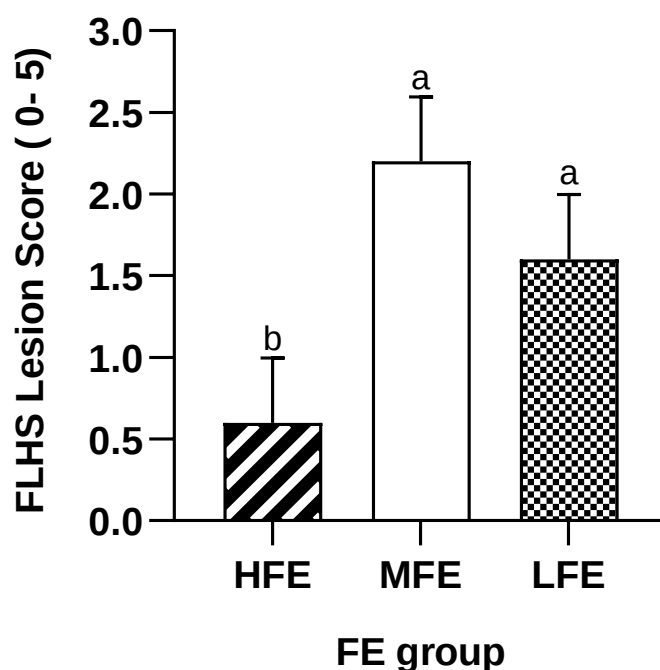


Figure 4.5 – Effect of varying feed efficiency on the physical gross appearance of livers of individually housed ISA Brown hens aged 45 weeks old. HFE: High Feed Efficiency; MFE: Medium Feed Efficiency; LFE: Low Feed Efficiency.

The FLHS lesion scoring of individual hens based on FE status are shown in Figure 4.6. Hens ranked as HFE had a lower lesions score (0.60 ± 0.40) when compared with both the MFE (2.20 ± 0.40) and LFE group (1.60 ± 0.40).



FE: Feed efficiency; FLHS: Fatty Liver Haemorrhagic Syndrome; HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency; SEM: Standard Error of Mean; abc Means within rows not sharing a common suffix are significantly different at the 5% level of probability.

Figure 4.6 – The effect of feed efficiency on FLHS lesions score as seen at necropsy of individually housed ISA Brown hens aged 45 weeks old of varying feed efficiencies (n = 30, 10 per FE group). Livers were scored on a scale of 0-5 where higher value indicates greater severity.

Correlations (r) between some liver characteristics and hen performance variables are presented in Table 4.4. The results showed that FLHS lesions score had a moderate positive correlation with slaughter weight ($r = 0.47$, $p = 0.01$). The FI, FCR, abdominal fat pad, liver weight and FLHS lesion score were seen to be positively correlated to liver fat % and liver TBARS. FLHS lesion score had strong positive correlation ($r = 0.73$, $p < 0.001$) with liver weight and a moderate association with abdominal fat pad weight ($r = 0.48$, $p < 0.01$).

Table 4.4 - Correlations (r) of liver tissue characteristics, organ parameters and performance traits of individually housed ISA Brown hens aged 45 weeks.

Traits	FLHS lesions score	Liver DM (%)	Liver fats (%)	Liver TBARS
BW – 45 weeks, g	0.47*	0.63***	0.70***	0.60***
FI, g	0.36	0.45*	0.50*	0.62***
FCR	0.34	0.39*	0.42*	0.86***
Liver weight, g	0.73***	0.49*	0.61***	0.26
Abdominal fat pad weight, g	0.48*	0.66***	0.71***	0.63***
FLHS lesions score	-	0.97***	0.77***	0.55**

BW: Body weight; FI: Feed intake; FLHS: Fatty liver haemorrhagic syndrome; FCR: feed conversion ratio; DM: Dry matter; TBARS: Thiobarbituric acid reactive substances. *: coefficients with a p value of ≤ 0.05 ; ** - $p < 0.01$; *** - $p < 0.001$

The microscopic examination of liver tissue from hens ranked as HFE and LFE are presented in Figure 4.7. The HFE liver tissues A1 and A2 were reported to have very little or no cytoplasmic lipid vacuoles compared to LFE hens (B1 and B2) that had swollen hepatocytes with distended cytoplasmic lipid vacuoles.

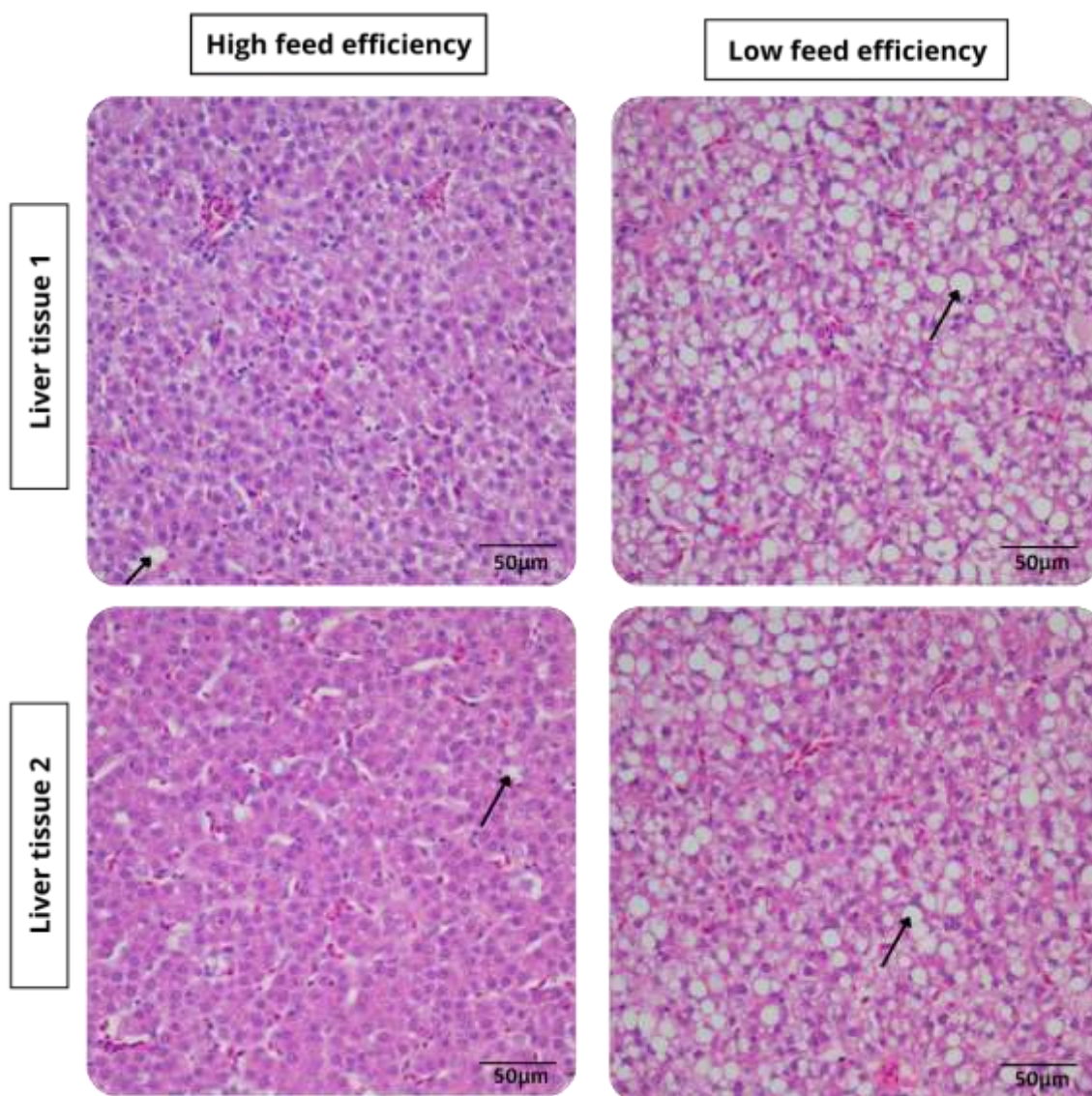


Figure 4.7 - Images of microscopic histological sections of liver tissue after H&E staining, showing liver cells (hepatocytes) of individually housed ISA Brown hens ranked based on feed efficiency. Liver samples were taken when hens were aged 45 weeks. Steatosis within the cytoplasm of the hepatocytes is identified with a black arrow.

4.5 Discussion

The objective of this study was to investigate the associations between performance, organ characteristics and prevalence of FLHS in individually housed hens aged 35 to 45 weeks, which were retrospectively ranked based on FCR during early, lay (25-30 weeks).

The lack of association between FI and egg production agrees with the findings from Harms et al (1982) and Lacin et al (2008) which suggests that feed consumption is not a driver of egg production. The lack of association between FI and egg weight suggests that hens, who consumed more feed, did not lay bigger eggs; instead, they were heavier in weight and had a poorer feed to egg conversion ability. On the contrary, the positive association between feed consumption and BW suggests that in inefficient hens, nutrients and energy which are not used for egg formation will be either excreted or used for BW gain and maintenance. This is in agreement with a previous research which suggested that at peak production changes in BW accounts for about 50-70% of the variation in feed intake (Fairfull & Chamvers, 1984), and flocks whose BW are managed around the breed standard or a recognized optimal weight for age have achieved significant improvements in production performance, with improved persistency of egg production (Parkinson et al., 2015). Variation in BW is an important determinant for the variation in feed intake and FE of laying hens. The heavier abdominal fat pad weight seen in the LFE hens and its positive associations with FI, BW at 45 weeks and FCR confirms that in inefficient hens, energy is not utilised for egg formation and body maintenance instead energy will be excreted as metabolic waste or heat output or stored as lipids in the abdominal cavity in form of visceral fat.

The weight of the internal organs and its contribution to the total BW reflects the health condition of animals (Smith et al., 2011). The higher liver mass seen in the HFE hens of the current study, illustrates that differences in the liver appear to be related to body weight and FI. This agrees with previous studies which show that the size of visceral organs are related to

the level of FI as the size of these organs may influence energy requirements for basal metabolism (Donald et al., 1990). The higher absolute weight of the gastrointestinal tract (GIT) of the LFE hens in the current study agrees with the results of Isabelle & Doreau (1995), which suggests that the GIT increases in size and thus has an increased energy requirement with increasing levels of feed intake, due to a greater volume of digesta and supply of nutrients in agreement with. However, when the weight of the GIT was corrected for BW in the current study, there was no difference seen in the mass of the gut across the three FE groups. Heavier gizzard improves nutrient utilisation in chickens, presumably due to a greater functionality (Moss et al., 2018). This may explain the increase in nutrient efficiency in the HFE hens. The gizzard has also been described as the pace-maker of gut motility which includes episodes of reverse peristalsis that increases the exposure of digesta in the gizzard, to proventricular secretions of digestive enzymes (Ferket, 2000). Further, larger well-functioning gizzards contribute to inhibiting feed overconsumption as it limits the quick passage of feed from the gizzard to the duodenum (Cline et al., 2010). Research has shown that satiety can be induced by stretch and muscular activity of the gizzard (Denbow, 1994) or through humoral signals such as ghrelin, gastrin and cholecystokinin (Richards & Proszkowiec-Weglarz, 2007). Another report showed that hyperphagia followed by poor FE within *ad libitum* fed broiler chickens selected for a high growth rate may only be possible when the gizzard is underdeveloped (O'Sullivan et al., 1992). Therefore, the higher appetite and subsequent FE seen in the LFE hens of the current study may be linked to the smaller gizzard size.

The colour of the liver is an important indicator for poultry viscera health as abnormalities in the colour may be caused by disease conditions (Chao et al., 1999). For example, the liver b* value – which gives an estimate of yellowness, where a higher value is more yellow – was greatest in LFE birds and corresponded to a higher hepatic fat deposition. According to King & Chen (1998), lipid accumulation in the hepatic cells of the chicken contribute to the intensity

of liver colour. Moreover, the studies by Shini & Bryden (2009) and Rozenboim et al (2016) indicated that a liver with a stronger yellow colour is associated with abundant fat reserves and the hallmarks of FLHS, which include subcapsular and parenchymal haematomas and haemorrhages.

The higher TBARS levels obtained in the LFE hens compared to both the HFE and MFE hens suggests higher oxidation in the liver tissue due to free radical exposure, which can cause damage to proteins, lipids, and DNA, and can contribute to cell toxicity and lead to the development of FLHS. Oxidative stress, an imbalance between production of free radicals and reactive oxygen species (ROS), leads to damage of important biomolecules and cells, and can potentially impact the whole organism (Ďuračková, 2009). Liver fat acts as a potential substrate for ROS toxicity and lipid peroxidation (Ucar et al., 2013). Reactive oxygen species cause lipid peroxidation and cytokine production, contributing to hepatocellular injury and fibrosis (Peeverill et al., 2014), and promote progression from simple steatosis to fatty liver disease (Leamy et al., 2013). A positive correlation between liver fats and liver TBARS indicates a higher risk of FLHS in the LFE hens due to the high levels of oxidative metabolites (TBARS). These results are supported by Rolo et al (2012) and Ucar et al (2013), who indicated that lipid peroxidation induces hepatic inflammation which leads to the development of diverse hepatic lesions associated with FLHS.

FLHS is a metabolic disease mostly observed in caged laying hens and associated with high egg production and positive energy balance. The higher FLHS lesion score observed in the LFE hens compared to the HFE hens, suggests that greater feed intake is associated with increased incidences of FLHS. Prolonged consumption of excess energy, such as that observed in the LFE hens' group of the current study suggests an increased fat synthesis by the liver, which can alter liver function and the FE of laying hens. The increased FLHS lesion score in the current study was also associated with greater BW, abdominal fat content, liver weight,

liver fat and dry matter content. This is supported by Trott et al (2013) who reported that approximately 97% of the FLHS affected birds were found to have large fat deposits or were obese. Similarly, King & Chen (1998) reported that a higher chance of FLHS is due to abnormal fat accumulation in the abdominal cavity, the visceral organs and liver cells of the chicken. The study by Whitehead (1975) also reported that increased lipid level of the FLHS birds of up to 47.7% could have a positive effect on dry matter content of the liver. The present study indicates that inefficient hens had greater fat deposition in the liver, thus increasing incidences of metabolic differences in liver function. The increased lipid availability was expressed in the livers of LFE hens by higher lipid peroxidation value, as well as swollen hepatocytes noted on microscopic examination of the liver tissue from LFE hens. The excessive accumulation of fat within the hepatocytes weakens the structural integrity of the liver, leading to haemorrhage.

4.6 Conclusion

The present study agrees with the hypothesis and concludes that there are important relationships between hen BW and FE with organ characteristics and FLHS prevalence in laying hens. Inefficient hens consumed more feed and stored excess energy beyond that required for egg production, which resulted in a positive energy balance leading to greater BW, higher abdominal fat deposition and higher incidences of FLHS lesion score compared to HFE hens. The LFE hens also had higher fat content in the liver, higher yellowness value, and enlarged and fat filled hepatocytes in the liver tissues compared to HFE hens. The result of this study shows that excessive BW in hens is associated with FHLS, and that excessive energy and nutrient intake may lead to compositional and biochemical changes in the liver which may impact liver function and health. Due to the tendency for hens to become overweight when allocated to *ad libitum* feeding regimes, the management of body weight around or slightly lower than the breed standards, especially in high producing flocks may assist in identifying flocks predisposed to the incidence of the FLHS and the prevention of this incidence. A better understanding of the role of oxidative stress and inflammatory response in the pathogenesis of FLHS would help in the development of new management strategies.

CHAPTER FIVE

5. Proteomics profile and bacteriostatic activity of albumen from hens exhibiting different extreme feed efficiencies.

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This is the fourth result chapter which examined the differences in the bacteriostatic activity and proteome composition of egg albumen from ISA Brown hens of varying FE. The hypothesis of this study was that hens maintain their FE status attained in early lay until late lay, and there were differences in the bacteriostatic and proteomics characterisation of albumen from hens of varying feed efficiencies.

5.1 Abstract

Previous studies showed that eggs from 40-week-old hens designated as having high feed efficiency had higher albumen height and Haugh unit. However, the persistency of this trend in the old hens as well as the proteomic profile and bacteriostatic potential of egg albumen from hens exhibiting various feed efficiency is not known. The hypothesis of this study was that albumen from old hens ranked as highly feed efficient hens had higher quality, improved antimicrobial properties and a unique proteomic signature. Based on feed to egg conversion ratio (FCR) status obtained in early lay, a total of 119 hens aged 70 weeks were grouped into high feed efficiency (HFE, FCR - 1.83, $n = 39$), medium feed efficiency (MFE, FCR - 2.05, $n = 41$) and low feed efficiency (LFE, FCR - 2.39, $n = 39$) and monitored for differences in hen performance, egg quality characteristics, bacteriostatic properties of the albumen against *Salmonella typhimurium* (ST) and *Staphylococcus aureus* (SA) and proteomic composition of the albumen. Albumen height and Haugh unit was greater ($p < 0.01$) in HFE hens compared to LFE hens. There was a greater zone of activity ($p < 0.05$) against ST and SA in the egg albumen from HFE hens (SA = 2.19 mm, ST = 2.09 mm) compared to the egg albumen from LFE hens (SA = 1.73 mm, ST = 1.66 mm). Using the Thermofisher Proteome Discoverer software, a total of 20 protein groups were detected in the albumen of eggs from HFE hens, and using the PEAKS Studio XPro software, two proteins - Gallinacin-11 and Actin 5, were significantly differentially expressed between albumen from HFE and LFE hen eggs. The study concludes that in late lay, albumen height and HU of hen eggs ranked as HFE was higher and had a potentially higher bacteriostatic defence compared to albumen from LFE hen eggs. This may be linked to the presence of additional antimicrobial peptides detected during the discovery proteomic mass spectrometry analysis pipelines.

Keywords: Layer hens, feed efficiencies, egg albumen, antibacterial properties, proteomics

5.2 Introduction

The chicken egg is a reservoir of nutrients, containing proteins, lipids and other nutrients which support embryo development and provides protection against microbial invasion. The egg albumen which constitutes up to 60% of the total egg weight (Kovacs-Nolan et al., 2005; Abeyrathne et al., 2013), is a highly viscous solution comprised of water, proteins and glycoproteins (Feeney et al., 1960, Mann, 2007). Its protein fractions includes major proteins – ovalbumin, conalbumin or ovotransferrin, ovomucoid, ovomucin, lysozyme, and minor proteins - avidin, cystatin, ovomucoid, ovoglobulins, ovoflavoprotein, and ovomacroglobulin (ovostatin) (Guha et al., 2018). These proteins have several functions including antimicrobial, enzymatic and anti-enzymatic, cell growth stimulatory, metal binding, vitamin binding, and immunological (Ibrahim, 1997, Guha et al., 2018), all targeted at acting as part of the natural defence system of the egg to inhibit bacteria growth and invasion.

The feed efficiency (FE) in laying flocks is a performance trait of economic importance, calculated as the rate of feed consumed to egg mass produced. Recent study has shown that FE varies considerably among individual 55 week old hens in a flock (Akter et al., 2018). The study of Anene et al (2020), demonstrated that these differences set in from the early stages of lay and are maintained up to 40 weeks of age (Anene et al., 2021). These studies also showed that variation in the feed efficiency of hens are associated with egg quality where hens with a lower (better) FCR, have a higher albumen height, Haugh unit and generally, better egg quality measurements (Akter et al., 2018; Anene et al., 2021). The height (thickness) of the egg albumen is an important indicator of egg freshness and the corresponding Haugh unit (HU), a correction of albumen height with egg weight, is a measure of quality in the egg industry. The study by Wang et al (2019), reported that albumen height is influenced by the presence of ovomucin and the quality of eggs could be positively associated with the amount of ovomucin present in the thick albumen. An earlier work by Toussant & Latshaw (1999) found that the

total amount of ovomucin isolated from the thick albumen of the eggs with higher HU was much higher than the amount isolated from the thick albumen with a lower HU. Taken together, this may suggest a linkage between hens with better feed to egg conversion efficiency having a higher HU and ovomucin content and thus presenting a higher bacteriostatic potential against microorganisms. As the egg industry strives to produce hens with laying life up to 100 weeks, it is important to understand how hens maintain uniform performance traits throughout their laying life, as variations can negatively affect hen productivity, external and internal egg quality characteristics, farmers profitability and egg safety for consumers (Roberts et al., 2015). Although several studies have reported on the antimicrobial activity of the egg albumen (Wellman-Labadie et al., 2008; Fang et al., 2012; Abdel-Shafi et al., 2016), to the best of our knowledge, there are no documented literature reports on the possible differences in the bacteriostatic abilities of egg white from old hens, based on their inherent variation in feed efficiency attained from early lay. Therefore, the objectives of this research were to investigate the variation in hen performance and egg quality of aged ISA Brown hens based on feed efficiencies attained in early lay, evaluate the differences in bacteriostatic effectiveness of egg white from these grouped hens and lastly, explore the differences in the proteome composition of their respective albumens following a discovery proteomics protocol.

5.3 Methodology

Ethics approval

The experiment was conducted at the Poultry Research Foundation (PRF) layer rearing facility, within The University of Sydney Camden Campus, New South Wales, Australia. The procedures and activities conducted in this study, were approved by the University of Sydney Animal Ethics Committee, protocol number AECL 2017/1212 and in accordance with the 8th edition of the Australian code for the care and use of animals for experimental purposes (NHMRC, 2013)

Experimental diet

A wheat soy mash diet containing 16.3% crude protein and 2750 kcal/kg of gross energy was formulated according to National Research Council, (NRC, 1994), to meet the nutritional requirements of the birds, as presented in Table 2.1. The same diet was supplied to all experimental birds in individual metal constructed feeding troughs placed in front of the cages. Fresh diets were measured out weekly and offered, and at the end of the week, feed not consumed was taken out, measured, and recorded.

Hen Performance

The hens used in this study were selected based on the protocol and results reported from the preliminary study (Anene et al., 2020). Briefly, 455 individually caged ISA Brown hens were monitored for six weeks from 25 until 30 weeks of age (early lay stage). At 30 weeks and based on feed to egg conversion ratio (FCR), the hens were ranked into quintiles and the hens in the first, third and fifth quintile were grouped into high feed efficiency (HFE), medium feed efficiency (MFE) and low feed efficiency (LFE). The HFE hens had an average FCR of 1.83; MFE, 2.05 and LFE, 2.39. At 70 weeks of age (estimated as the late laying stage), a total of 119 hens ($n = 39$ hens in the HFE and LFE group, and $n = 41$ in the MFE group) were randomly assigned to the three FE groups. Each hen was housed individually in 3-tier layer metal cages measuring $25 \times 50 \times 50$ cm per cage. The experiment lasted for 42 days, from when the hens were 70 weeks until 75 weeks. Hens were weighed at 70 weeks using an electronic scale with a digital output and recorded as initial body weight (IBW) and again at 75 weeks, recorded as final body weight (FBW). The difference in weight at the end of the experimental period was recorded as the body weight change (BWC). Daily egg production (EP) was recorded and calculated in percentages as $(\text{total number of eggs produced in seven days} \times 100) / 7$ (number of days of the week), egg weights were recorded daily per hen in grams using a digital weighing balance, average daily feed intake (ADFI) was calculated as $(\text{weekly feed offered} - \text{weekly feed unconsumed}) / 7$ (number of days in the week), average egg mass per hen in grams was calculated as: $EP \times EW / 100$; where EP = Egg production, and EW = average egg weight, and the feed (to egg) conversion ratio (FCR) was calculated as the grams of feed consumed per gram of egg mass. Egg quality analysis was carried out as described in Chapter 3.

Egg albumen proteomics

Protein identification of albumen samples by mass spectrometry analysis was performed at the Sydney Mass Spectrometry, a core research facility at the University of Sydney, Australia. A

total of 10 egg whites were collected from 75-week-old hens in the HFE and LFE groups ($n = 5$ per FE group). Egg albumen was carefully separated from the yolk, collected in a sterile container, and weighed. Albumen was then transferred to a clean and dry beaker, and using a magnetic stirrer at a low speed, albumen was homogenised without foaming. After homogenising to attain a uniform consistency, albumen was collected in tubes and stored at -80°C . The albumen samples were then prepped, and discovery proteomics analysis was carried out according to the following protocol.

Sample preparation

The frozen albumen was allowed to thaw on the bench and 1ml of albumen was transferred into labelled tubes. Sodium deoxycholate (SDC) lysis buffer was prepped using 400 mg of sodium deoxycholate, 1 ml of 1 Molar stock of ammonium bicarbonate and 10 mls of MilliQ water. This was then vortexed to mix thoroughly. A total of 950 μL of the SDC lysis buffer was pipetted into labelled tubes and 50 μL aliquot of albumen samples was added and vortexed to mix thoroughly. Five mls of the sample from the above step was mixed to 20 mls of MilliQ water and vortexed. The Bovine Serum albumin (BSA) standard was prepared using 0.4 mg of sodium chloride and 5 mls of MilliQ water. Using the BSA serum and the SDC buffer, the protein standards were serially diluted from 2000 $\mu\text{g/mL}$, until blank. 10 μL of the standards and samples were then transferred to microplates in doubles and 200 μL of BCA protein assay pipetted unto standards and samples. The elution buffer that was used, was prepared as follows: elution buffer A containing 5 mM K_2HPO_4 in 20% (v/v) acetonitrile, at pH 3.0 while elution buffer B containing 5 mM K_2HPO_4 in 20% (v/v) acetonitrile, 350 mM KCl at pH 3.0. The microplate was incubated at 37°C for 30 minutes. Microplate was then read in a spectrophotometer to calculate the absorbance, concentration, and slope of standards. A total of 50 μg of stock solution and 50 μL of buffer was transferred into a tube. This was vortexed and 5 μL of reduction and alkylation buffer was added. This mixture was centrifuged for one

minute and placed on a silicon holder to incubate in a water bath at 40 – 45 °C for 10 – 15 minutes. Samples were removed from incubator and 220 µl of ice-cold acetone was pipetted into the tubes to precipitate out the protein. Tubes were vortexed to mix thoroughly and then placed in -20°C for 60 minutes. This step was important as it enabled the concentration of the protein sample as well as the removal of undesirable substances. 1 ml of Acetonitrile, 500 ml of Triethylammonium bicarbonate and 8.5 mls of MilliQ water was used to prepare a trypsin digestion buffer and vortexed. The trypsin vial was resuspended using 40 µl of the trypsin resuspension buffer (acetic acid) and pipetted several times to mix thoroughly. The samples were then removed from -20°C, vortexed and centrifuged for 10 minutes. The supernatant was carefully decanted, and the tube left open to dry the excess acetone from the protein pellet. The trypsin digestion solution was then prepared using 500 µl of trypsin digestion buffer and 10 µl of the resuspended trypsin. After checking that the acetone had evaporated completely, 50 µl of digestion solution and the resuspension trypsin was added to the dried samples. This was then vortexed thoroughly to resuspend completely and set in a platform shaker for 10 – 15 minutes. The samples were then incubated overnight at 37°C and then 10 µls of formic acid was added to the samples, vortexed, spined in the centrifuge and set in the speed vacuum for two hours to dry off the liquid. This allowed the proteins to settle on the walls of the tubes. A loading buffer was prepared using 0.1% formic acid and 3% acetonitrile. 100 µls of forming buffer was added to the samples and was set in silicon tubes and placed in the sonicator for 5 minutes.

Mass Spectrometry

Peptides were resuspended in 50ul 3% (v/v) acetonitrile/0.1% (v/v) formic acid, briefly sonicated and centrifuged at 16,000g for 5 min. Samples were separated by nano-LC using an Ultimate 3000 HPLC and autosampler system (Dionex, Amsterdam, Netherlands) coupled to an in-house built fritless nano 75 µm × 30 cm column packed with ReproSil Pur 120 C18

stationary phase (1.9 μm , Dr, Maisch GmbH, Germany). Liquid chromatography (LC) mobile phase buffers were comprised of A: 0.1% (v/v) formic acid and B: 80% (v/v) acetonitrile/0.1% (v/v) formic acid. Peptides were eluted using a linear gradient of 5% B to 40% B over 100 mins and then 95% B wash over 1 min at a flow rate of 250 nL/min. The LC was coupled to a QExactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Scoresby). Column voltage was 2300 V and the heated capillary set to 275 °C. Positive ions were generated by electrospray and the Orbitrap operated in data-dependent acquisition mode. A survey scan of 300-1600 m/z was acquired (resolution = 70,000, with an accumulation target value of 3,000,000 ions). Up to 15 of the most abundant ions ($>1.7\text{e}5$ ions), with charge states $\geq +2$ were sequentially isolated and fragmented and target value of 100,000 ions collected. Ions selected for MS/MS were dynamically excluded for 20s.

Data interpretation and quantification

For data processing, Proteome Discoverer software 2.4 (Thermo Fisher Scientific, Waltham, MA, USA), was used to interpret raw data files produced by mass spectrometry. The Gallus gallus SwissProt protein database (vr2017-20.25) was used in combination with a common contaminants database. The parameters for database searching were as follows: trypsin was selected as the enzyme; two missed cleavages were allowed at maximum; precursor mass tolerance was set to 10 ppm; fragment mass tolerance was set to 0.05 Da; carbamidomethylation of cysteine; methionine oxidation and acetyl (protein N-terminal) were allowed as dynamic modification. The results were filtered at a 1% FDR. The minor feature detection was used to provide the relative quantitation of the observed peptides. Additionally, the raw data produced by mass spectrometry, was re-interrogated using PEAKS Studio XPro label free quantitative module for a relative yet quantitative comparison. The output report

significance parameters were set as follows: Protein fold change was ≥ 2 , Protein significance was ≥ 20 ; Mass Error Tolerance was set at 15.0 ppm and the FDR Threshold was set at 1%.

Microbiology assay

The bacterial inhibition properties of egg albumen from hens of varying feed efficiencies were tested against gram-negative *Salmonella typhimurium* and gram-positive *Staphylococcus aureus* following the Kirby–Bauer standard disc diffusion assay (Bauer et al., 1966) that is the lowest concentration of anti-microbial material ($\mu\text{g}/\text{disc}$) that will inhibit the visible growth of a microorganism after overnight incubation. Pathogenic *Salmonella typhimurium* (ATCC 143) and pathogenic *Staphylococcus aureus* (ATCC 25933) which were frozen at $-20\text{ }^{\circ}\text{C}$ in glycerol, were obtained from the Veterinary Teaching Hospital of the University of Sydney, Camden Campus Australia. The bacteria were cultured on sheep blood agar plate and incubated at $37\text{ }^{\circ}\text{C}$ for 24 hours, and they were then maintained at $4\text{ }^{\circ}\text{C}$, sub-culturing every 4 weeks. To prepare the bacterial inoculum, two colonies of the bacteria from the sheep blood plate were collected using a sterile loop and transferred into a tube containing 5mls of phosphate buffer saline. This was serially diluted up till 2^{-4} CFU/mls and the inoculum was utilised within 15 minutes of preparation. A total of 22 eggs from the LFE and HFE groups ($n = 11$ per FE group) collected from the experimental flock when the hens were 98 weeks old, hand broken, and the whites separated manually from the yolk. Using a magnetic stirrer, the thin and thick albumen per egg was individually homogenised gently for 30 min on slow speed, to reduce the viscosity and to avoid foaming. The serially diluted bacterial inoculum was then transferred to the pre-sectioned Mueller Hilton plates which have been labelled accordingly as control, high, medium, and low, representing egg albumen from high, medium, and low feed efficient hens. The inoculum was allowed to marinate on the plates for 20 seconds and the excess was discarded. Blank paper discs were soaked in the homogenised albumen from the hens of

varying feed efficiencies for 15 seconds and using a sterile tweezer, the albumen impregnated discs was then carefully placed in the pre-sectioned plate, pressing the disc gently unto the plate and ensuring that the disc touches only one part of the plate. A negative control, blank disc soaked in phosphate buffer saline, was placed in the fourth section, marked as control. All sections were properly labelled, and plates were incubated at 37 °C for 24 hours. The zone of inhibition was observed, and the diameters measured using a transparent metric ruler. The bacterial plates were then subjected to light electron microscopy analysis using an Olympus light microscope. Magnification powers were set at 200µm and X10 for both *Salmonella typhimurium* and *Staphylococcus aureus* and micrograph images were captured. Measurements were taken consistently at four locations on each disc; the averages of the three replicates per egg were calculated.

Statistical analysis

The individually caged laying hen served as the experimental unit. Experimental data were analysed using the generalised linear model procedure of SAS (SAS Institute) with the FE group as the main effect. The student's test was performed between the HFE and LFE groups for microbiology data using the proc means procedure of SAS. All data are presented as least square means $\pm$ standard error of the mean (SEM). Differences among least squares means were computed using the pdiff procedure in SAS. The probability value denoting statistical significance was $p < 0.05$.

5.4 Results

Hen performance

The performance of individual hens between 70 to 75 weeks of age from varying FE groups are presented in Table 5.1. Briefly, FCR in the HFE and MFE hens was lower compared to the LFE hens. rankings obtained from early lay up to 75 weeks of age ($p < 0.001$). Hens ranked as HFE consumed less feed ($p = 0.0003$), had a higher egg weight ($p = 0.019$) and had a lower final body weight ($p < 0.001$), compared to the LFE hen group. There was no difference in egg production across the FE groups.

Table 5.1 - Average performance variables of individually caged ISA Brown hens ranked based on FCR aged 70 to 75 weeks.

Hen performance variables

FE group	FCR	EP	EW	EM	ADFI	IBW	FBW	BWC
HFE	1.93 ^a	91.7	67.5 ^a	61.8	118.6 ^a	2081 ^a	2153 ^a	71
MFE	1.98 ^a	92.5	64.9 ^b	59.9	118.8 ^a	2132 ^a	2204 ^a	77
LFE	2.16 ^b	91.6	65.3 ^b	59.6	128.6 ^b	2300 ^b	2402 ^b	101
SEM	0.048	1.38	0.70	0.92	1.87	35.3	40.3	19.6
<i>p</i> -Value	<0.001	0.889	0.019	0.202	0.0003	<0.001	<0.001	0.514

FE: Feed efficiency; HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency. SEM: Standard Error Mean. abc: Means within columns with a different superscript are significantly different at the 5% level of significance. EP: Egg produced per hen per day (%); EW: Egg weight (g); EM: Egg mass (g); ADFI: Average daily feed intake (g); FCR: Feed to egg conversion ratio = ADFI/Egg mass; IBW: Initial Body Weight (g); FBW: Final Body Weight (g); BWC: Body weight change (g); Egg mass = Egg weight × egg production/100 (g).

Egg quality

The albumen, yolk, and shell quality characteristics of eggs from hens of the different FE groups between 70 to 75 weeks are presented in Table 5.2. In brief, albumen height, Haugh unit and albumen:yolk ratio was greater in the HFE hen cohort, compared to the LFE groups ($p < 0.05$). The yolk width, yolk weight percentage and the yolk pH were however higher in the LFE hens compared to the HFE hens ($p < 0.05$). No differences were seen in the shape index and shell quality parameters across the three hen groups.

Table 5.2 - Internal and external egg quality characteristics from individually caged ISA Brown hens between 70 to 75 weeks of age, ranked based on feed efficiency.

Egg quality traits	Hen feed efficiency group ($n = 15$ per FE group)				
	HFE	MFE	LFE	SEM	p-value
Egg weight (g)	65.7	65.7	65.4	1.02	0.97
Egg width (mm)	44.6	44.4	44.3	0.25	0.66
Egg height (mm)	58.4	59.1	58.4	0.52	0.61
Shape index %	76.5	75.3	76.0	0.67	0.47
Albumen height (mm)	8.9 ^a	8.3 ^a	7.3 ^b	0.25	0.001
Albumen width (mm)	74.4	73.3	75.4	1.22	0.48
Albumen index %	12.1 ^a	11.4 ^a	9.7 ^b	0.50	0.006
Albumen weight (g)	37.9	38.4	37.1	0.81	0.52
Albumen weight (%)	57.7	58.5	56.7	0.67	0.20
Haugh unit	92.6 ^a	89.8 ^a	83.6 ^b	1.39	0.001
Albumen pH	8.51	8.57	8.54	0.02	0.36
Yolk height (mm)	15.9	15.9	16.6	0.23	0.06
Yolk width (mm)	41.0 ^a	40.8 ^a	42.5 ^b	0.46	0.03

Yolk index %	38.7	39.1	39.2	0.59	0.84
Yolk weight (g)	17.1	17.3	18.0	0.34	0.17
Yolk weight (%)	26.1 ^a	26.4 ^a	27.6 ^b	0.38	0.02
Yolk pH	6.05 ^a	6.13 ^{ab}	6.17 ^b	0.02	0.002
Albumen:yolk ratio	2.22 ^a	2.21 ^a	2.05 ^b	0.04	0.03
Yolk colour	11.4 ^a	10.7 ^b	11.2 ^{ab}	0.18	0.03
Shell weight (g)	6.35	6.33	6.17	0.11	0.48
Shell thickness (mm)	0.47	0.48	0.48	0.01	0.79
Shell weight (%)	9.66	9.65	9.46	0.16	0.61
Shell breaking strength (g)	4102	3933	3971	98.9	0.45

HFE: High feed efficiency; MFE: Medium feed efficiency; LFE: Low feed efficiency; SEM: Standard Error of Mean; abc: Means within columns with different superscript are significantly different at the 5% level of significance. Egg shape index = egg width/ egg length $\times$ 100; Albumen percentage = albumen weight/ egg weight $\times$ 100; Albumen index = albumen height/ (albumen width + albumen height /2) $\times$ 100 Yolk percentage = Yolk weight/ egg weight $\times$ 100; Yolk index = Yolk height/ yolk width $\times$ 100; Yolk:Albumen ratio = Yolk weight/ Albumen weight; Shell percentage = Shell weight/ egg weight $\times$ 100.

Egg albumen proteomics

The relative abundance of proteins present in egg albumen from hens of varying feed efficiencies was determined using a label-free quantitative proteomic approach. A total of 129 proteins were identified from egg white, out of which 120 were classified as high abundance including proteins such as ovalbumin, ovotransferrin, ovomucin, ovoinhibitor, lysozyme. Four proteins were classified as medium abundance and five proteins were in low abundance in the albumen samples, as presented in Table 5.3.

Table 5.3 - Proteins identified by HPLC mass spectrometry

Protein FDF ^a	Accession ^b	Description ^c	Coverage ^d	Peptides ^e	MW [kDa] ^f	Calc.pI ^g
High	P02789	Ovotransferrin	90.78	147	77.7	7.12
High	Q98UI9	Mucin-5B	67.84	161	233.4	5.60
High	P01012	Ovalbumin	100	67	42.9	5.29
High	P10184	Ovoinhibitor	75.42	47	51.9	6.58
High	P01005	Ovomucoid	85.24	24	22.6	4.91
High	P00698	Lysozyme C	87.07	27	16.2	9.07
Medium	P13648	Lamin-A	1.22	1	73.1	6.95
Medium	P14732	Lamin-B2	1.33	1	67.9	5.38
Medium	Q9W6V2	Neuronal growth regulator 1	2.27	1	37.9	7.03
Medium	Q5ZMH6	Methyltransferase-like protein 9	5.30	1	37.0	6.64
Low	P28686	CCN family member 3	3.42	1	38.2	7.42
Low	Q07496	Ephrin type-A receptor 4	0.71	1	109.4	6.79
Low	Q5ZMJ7	Protein CIP2A homolog	1.21	1	101.7	6.30
Low	Q90629	Trypsin II-P29	2.42	1	26.6	4.78
Low	P47832	60S ribosomal protein L26	4.69	1	15.2	10.36

a -The level of confidence or abundance for the identified protein; b- The Accession numbers of differentially expressed proteins according to the NCBIInr database; c- The description of the protein extracted from the search sequenced database; d - The percentage of the sequence covered by identification (PSMs only) from the included searches; e-The total number of distinct peptide sequences identified for the protein; f,g - The theoretical molecular weights and isoelectric points of the identified proteins were retrieved from the NCBIInr protein database.

As presented in Figure 5.1, 79 protein groups were found in the albumen samples from both HFE and LFE hens, however, an additional 20 protein group were detected in albumen from the HFE hens and were not found in the albumen samples from the LFE hens.

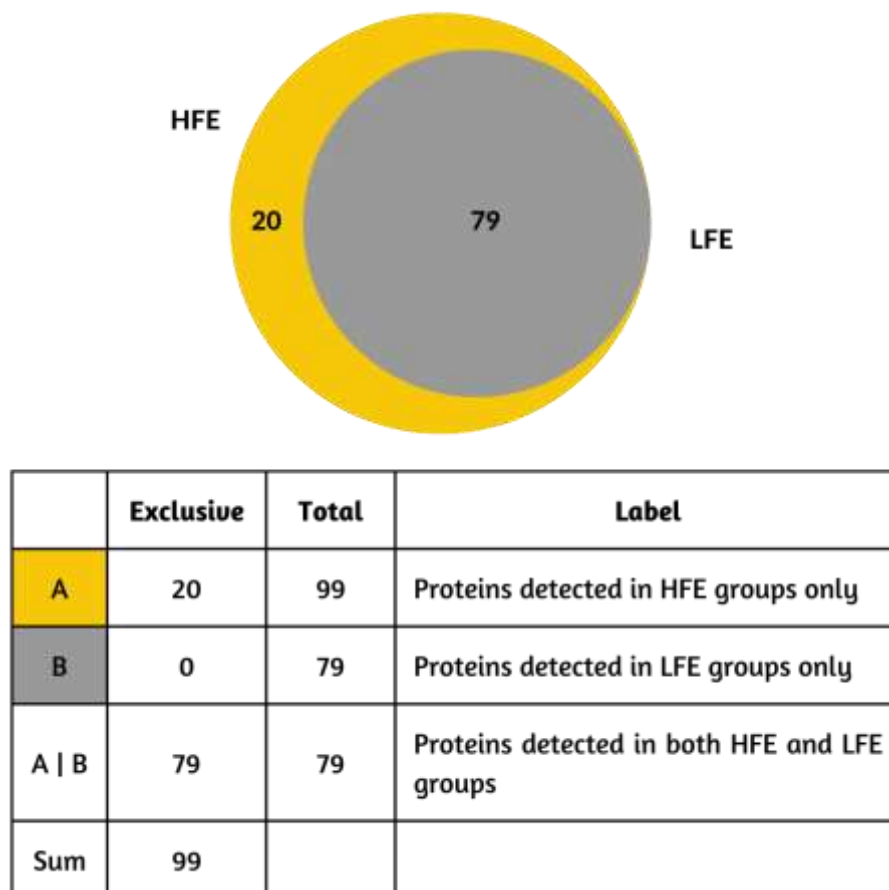
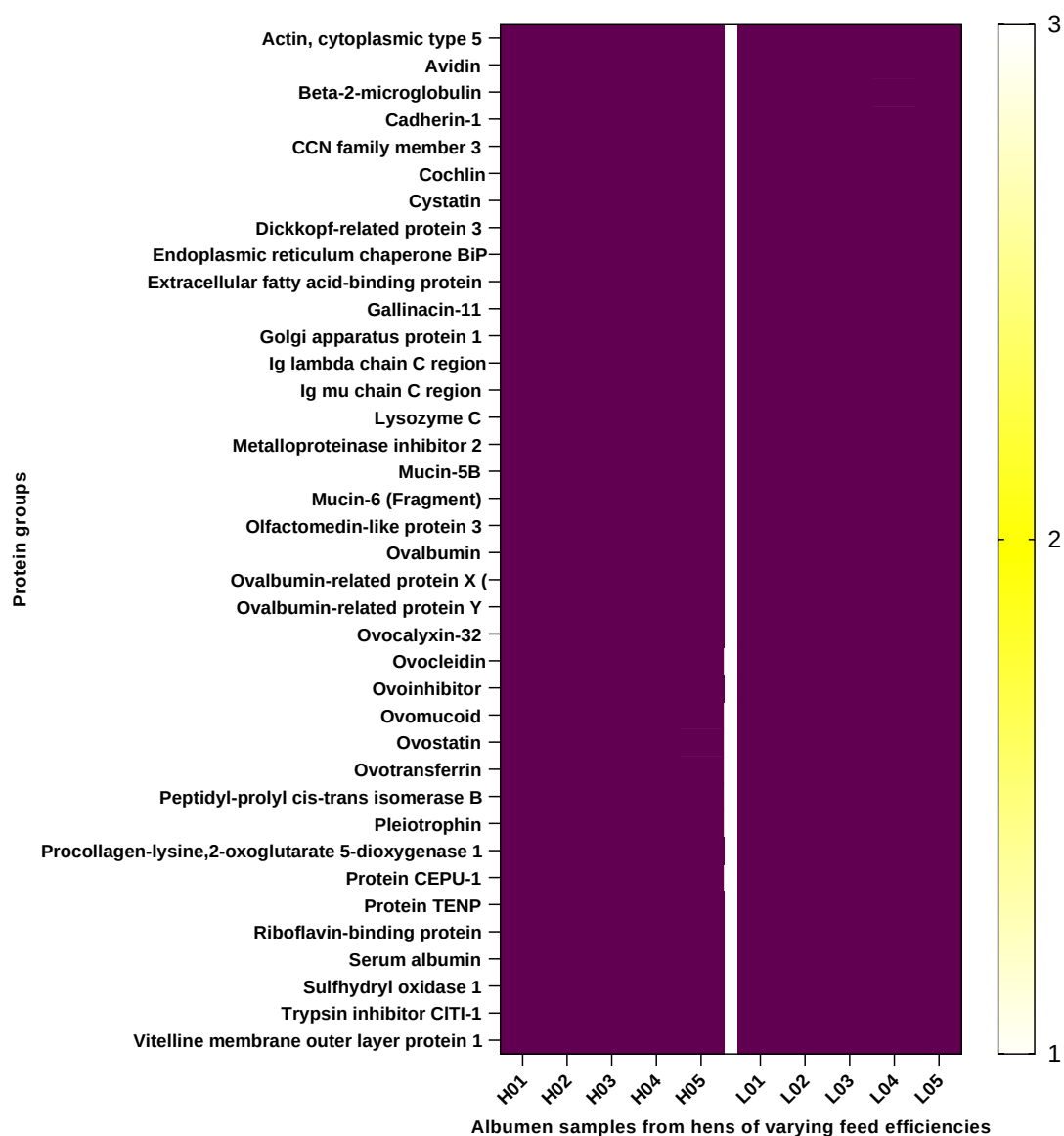


Figure 5.1 – The difference in number of protein groups that were detected in egg albumen collected from both high and low feed efficient ISA Brown hens aged 75 weeks old.

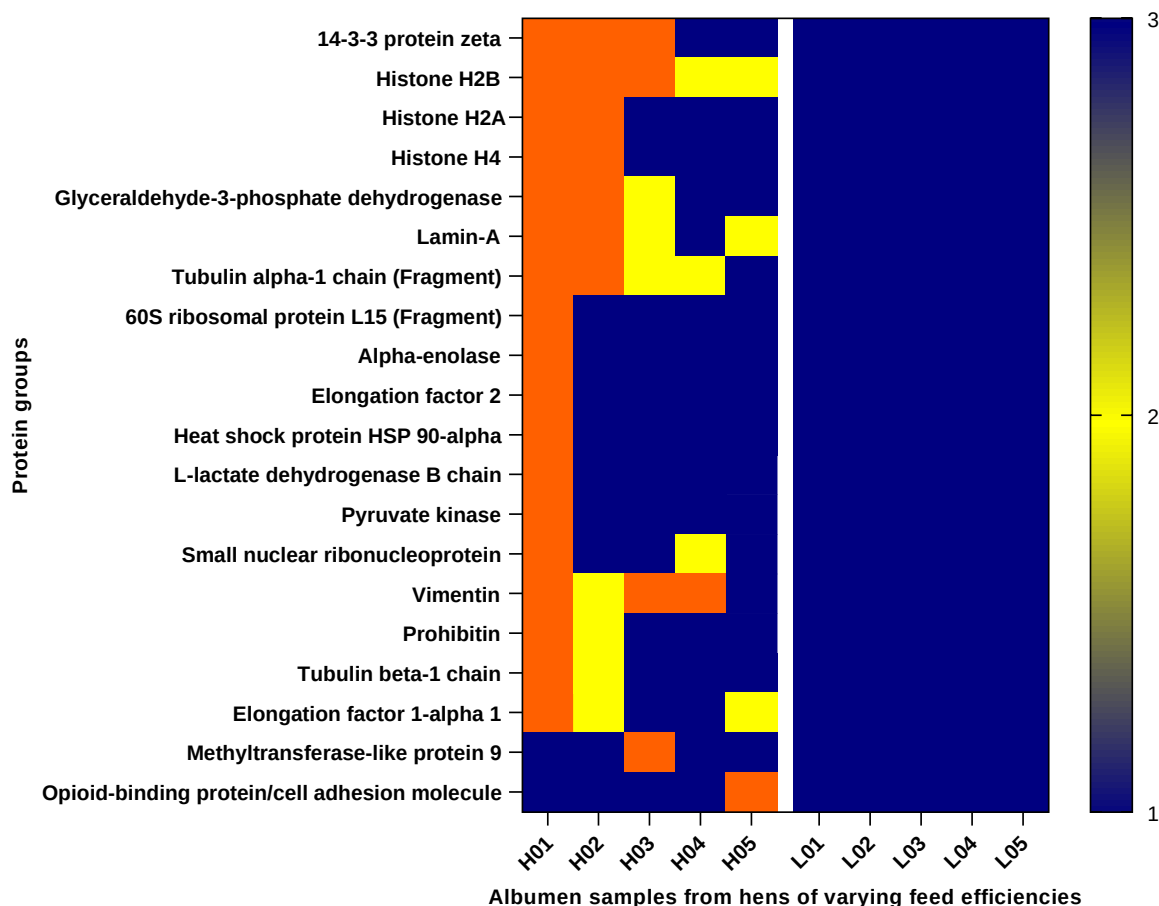
Figure 5.2 presents the heat map of the 38 protein groups were which detected in high abundance in both the HFE and LFE albumen samples. Antimicrobial proteins like lysozymes, ovalbumin, ovomucoid, ovocleidin were all found in high abundance in albumen from both HFE and LFE hens.



H01 – H05: Albumen samples from high feed efficient hens. L01 – L05: Albumen samples from low fed efficient hens. 1 – Protein group not found. 2 – Protein group detected. 3 – Protein group found in high abundance.

Figure 5.2 - Heat map showing the presence of 38 peptide groups that were detected in egg albumen collected from both high and low feed efficient ISA Brown hens aged 75 weeks old.

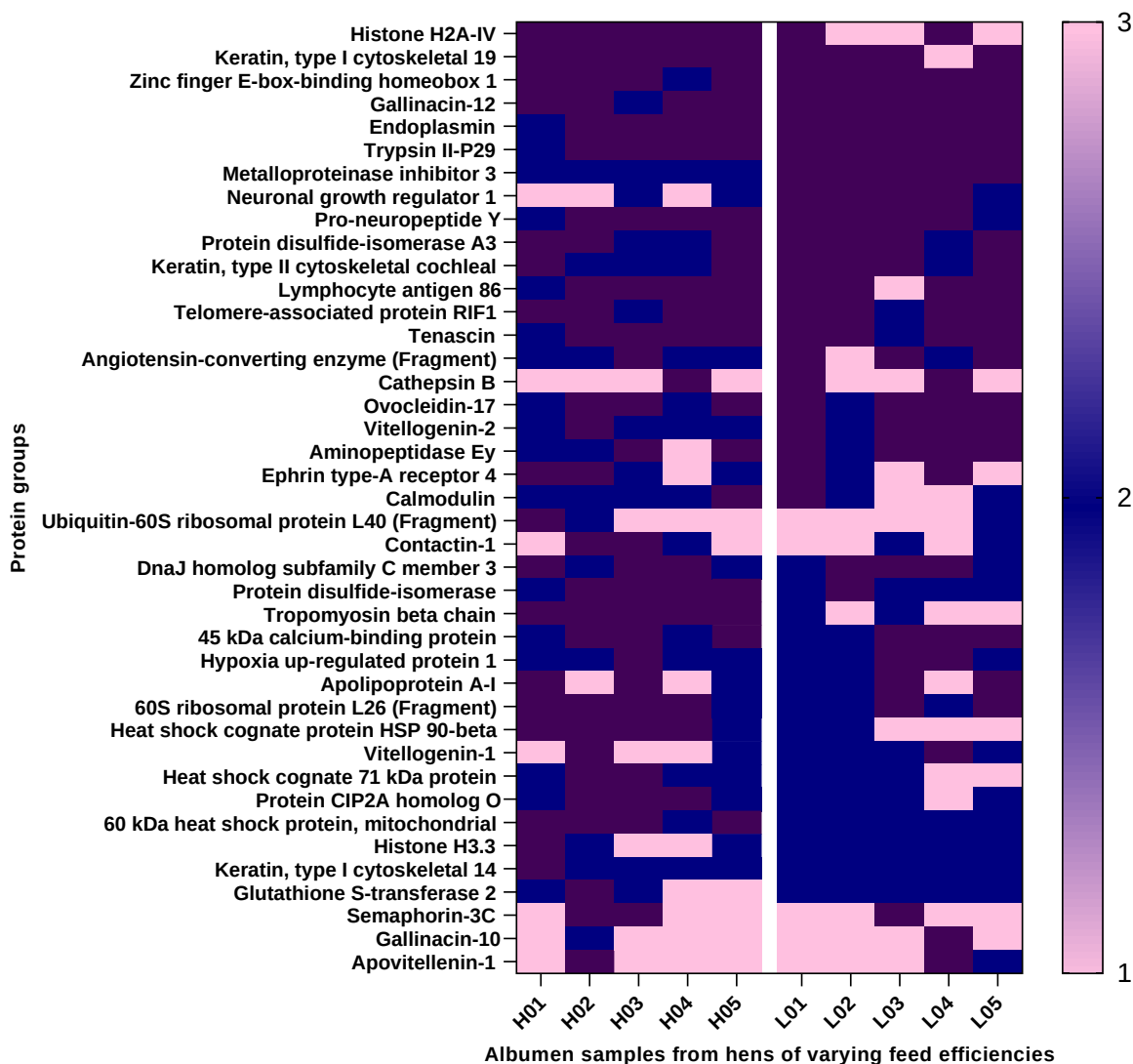
As presented in Figure 5.3, an additional 20 protein groups were found in high abundance in at least one sample of albumen from the HFE hens but not found or peaked in the albumen from LFE hens. Protein groups Vimentin, Histone H2B and 14-3-3 protein zeta was found in high abundance in three HFE samples while protein groups Lamin, Glyceraldehyde-3-phosphate dehydrogenase were found in two HFE albumen samples.



H01 – H05: Albumen samples from high feed efficient hens. L01 – L05: Albumen samples from low fed efficient hens. 1 – Protein group not found. 2 – Protein group detected. 3 – Protein group found in high abundance.

Figure 5.3 - Heat map showing the 20 protein groups were found in at least one sample of albumen from the HFE hens but not found or peaked in the albumen from LFE hens.

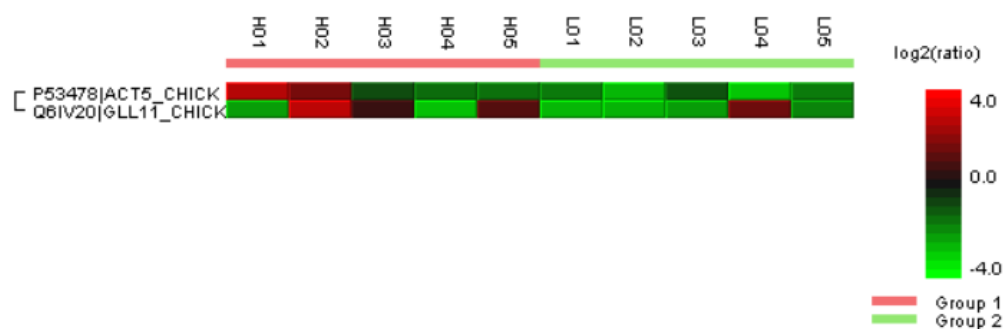
Figure 5.4 shows the remaining 41 protein groups which were found in either or both albumen samples. Histone H2A-IV was detected in high abundance in all HFE samples but was seen in high abundance in two and not found in three LFE samples.



H01 – H05: Albumen samples from high feed efficient hens. L01 – L05: Albumen samples from low fed efficient hens. 1 – Protein group not found. 2 – Protein group detected. 3 – Protein group found in high abundance.

Figure 5.4 - Heat map showing the distribution of 41 peptide groups that were detected in egg albumen collected from 75-week-old hens with high and low feed efficiency.

The heat map showing the proteins differentially expressed using the PEAKS Studio XPro label free quantitative module for a relative, yet quantitative comparison on the aforementioned stringency parameters is presented in Figure 5.5. Interestingly, two proteins - Gallinacin-11 and Actin cytoplasmic type 5,1 or 2 (which were expressed in high abundance in both HFE and LFE samples using the Proteome Discoverer Software), were differentially expressed in the albumen from the HFE hens compared to the albumen from LFE hens.



H01 – H05: Albumen samples from high feed efficient hens. L01 – L05: Albumen samples from low fed efficient hens. GLL 11 - Gallinacin-11; ACT5 – Actin cytoplasmic type 5

Figure 5.5 - A heat map, showing the expression of Gallinacin-11 and Actin in the albumen of eggs from hens ranked as high feed efficiency and low feed efficiency.

Egg albumen microbiology

The zones of bacteriostatic activity induced by the albumen of individual ISA Brown hens of varying feed efficiencies against *Salmonella typhimurium* and *Staphylococcus aureus* bacterial strains are presented in Table 5.4, while the light microscopy images of zones of bacteriostatic activity for *Salmonella typhimurium* are presented in Figure 5.6 and *Staphylococcus aureus* in Figure 5.7. Briefly, the homogenised albumen from HFE hens formed a greater zone of bacteriostatic activity against *Salmonella typhimurium* ($p = 0.04$) and *Staphylococcus aureus* and ($p = 0.03$) compared to the albumen from the LFE hens.

Table 5.4 - Average zone of bacteriostatic activity (mm) of egg albumen from individual ISA Brown hens of varying feed efficiencies against *Salmonella typhimurium* and *Staphylococcus aureus*.

	Bacterial strain	
	<i>Salmonella typhimurium</i>	<i>Staphylococcus aureus</i>
HFE	2.09	2.14
LFE	1.66	1.74
SEM	0.110	0.099
P-value	0.04	0.03

FE: Feed efficiency; HFE: High feed efficiency; LFE: Low feed efficiency. SEM: Standard Error Mean

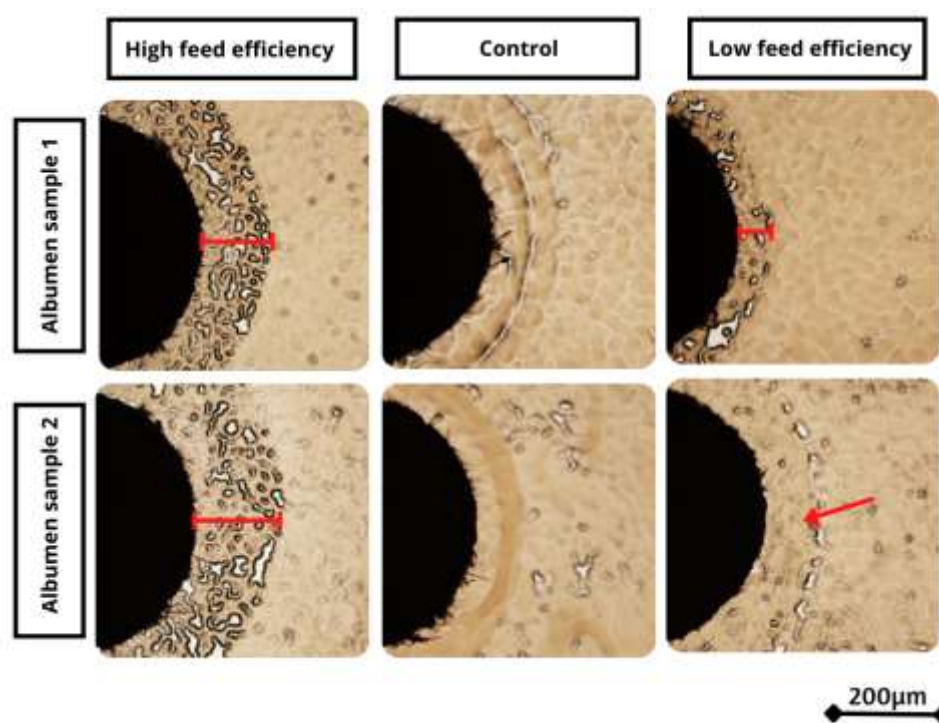


Figure 5.6 - Light electron microscopy images showing the zones of bacteriostatic activity of *Salmonella typhimurium* treated with egg white collected from individually housed ISA Brown hens of high and low feed efficiency at 37 °C for 24 h.

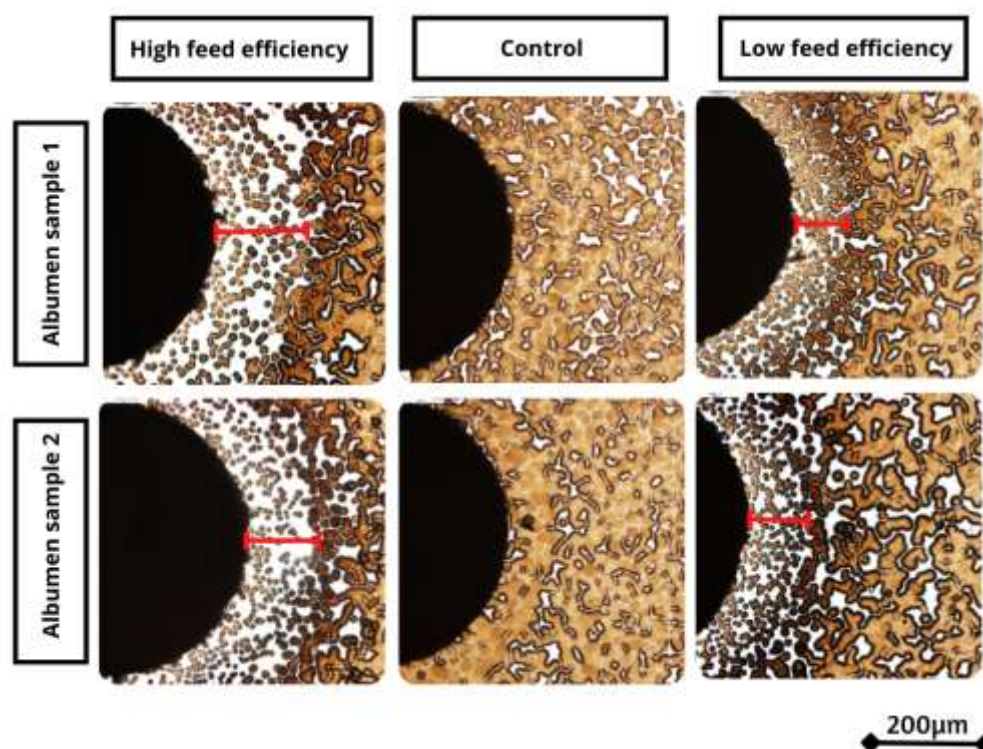


Figure 5.7 - Light electron microscopy images showing the zones of bacteriostatic activity of *Staphylococcus aureus* treated with egg white collected from individually housed ISA Brown hens of high and low feed efficiency at 37 °C for 24 h.

5.5 Discussion

Hens that were ranked on the basis of FE in early lay continued to have different FE at 75 weeks of age. HFE hens had a significantly lower (better) FCR than LFE hens and these differences were driven primarily by variation in feed consumption and not the egg mass. The improved feed efficiencies observed for HFE hens and the fact that all treatment groups produced similar egg numbers and egg masses, suggests a lower maintenance requirement and better feed utilization for hens in the HFE group, an important determinant for improved hen productivity and farmers profitability. The initial and final body weight was significantly different across the extreme FE groups, with hens in the LFE cohort weighing 9.5% and 10.3% more than hens in the HFE groups at 70 and 75 weeks respectively. The lack of difference in the rate of lay from hens of the HFE and LFE groups suggests that increased feed intake and higher hen body weight did not influence an increase in egg production.

The higher albumen height and Haugh unit of eggs from the HFE hens in this study may suggest a more viscous egg albumen and potentially, a stronger defence against bacterial invasion, compared to eggs from the LFE group. This is similar to the findings from (Wang et al., 2019) who reported a significantly positive association between Haugh unit and ovomucin content. Ovomucin is the most important egg albumen protein which is responsible for the thickness and structure of the inner thick egg albumen which plays a critical role in preventing bacterial movement within the egg white (Toussant & Latshaw, 1999; Strixner & Kulozik, 2011). The albumen index is the condition and coverage of the inner thick albumen which is an accepted measure of quality (Heiman & Carver, 1936). It is determined from the albumen height and width, where a higher albumen height and narrower width will suggest a higher egg quality. The higher albumen index obtained in the HFE group of the current study suggests a well-rounded coverage of thick albumen around the yolk, ensuring maximum protection of the egg yolk against physical shocks and bacterial penetration. In the current study with older hens, the

significantly higher yolk width and yolk weight percentage observed in the eggs from LFE hens may be linked to the body weight of hens in agreement with (Pérez-Bonilla, Novoa, et al., 2012) who reported that in their study eggs from the heavier hens had a higher proportion of yolk than eggs from the lighter hens. An increased yolk weight might also be an indication of a reduction in the strength of the vitelline membrane resulting easier permeation of moisture from the albumen especially during storage, and also in a higher occurrence of yolk rupture (Réhault-Godbert et al., 2019). The yolk pH in freshly laid eggs is generally about 6.0, but with extended storage the alkalinity increases to between 6.4 and 6.9 (Stadelman & Cotterill, 1995; Tânia et al., 2010; Feddern et al., 2017). The slightly higher yolk pH of 6.17 observed in the fresh LFE eggs in this study compared to that from the HFE - 6.05, suggests a difference in egg quality, even as fresh eggs. A more alkaline pH will negatively influence the vitelline membrane as albumen alkaline ions could be exchanged with yolk H^+ ions, leading to protein denaturation in the albumen and a weaker antimicrobial defence of the egg as a whole (Tânia et al., 2010). The colour of the yolk is a distinct measure of preference for consumers is influenced largely by hen age (Banaszewska et al., 2019; Krawczyk et al., 2020) the diet and laying rate of the hen (Nys, 2000). However, in the current study, we found a significantly higher yolk colour score in the HFE hens compared to the LFE hens even though feed composition was the same for all hens and the rate of lay was not different across FE groups. This was not expected, but might be may be linked to some water penetration from the albumen into the yolk, similar to the findings from of Perić *et al* (2017) who reported as the vitelline membrane weakens, water from the albumen penetrates the egg yolk to lighten the yolk pigments.

Using the PEAKS Studio XPro software - label free quantification module, two proteins (Gallinacin-11 and Actin cytoplasmic type 5,1 or 2) were significantly differentially expressed in the albumen from the HFE hens compared to that from the LFE hens. Gallinacin-11 (Gal-

11), the functional equivalent of beta-defensin in chickens, is an antimicrobial peptide that is found in the outer membrane of the vitelline layer of the egg. Gal-11 is rich in histidine, lysine, and arginine and has antibiotic, antimicrobial and defensin properties against gram-positive and gram negative bacteria by blocking the bacterial DNA replication and protein synthesis in bacteria causing death of the microbe (Sugiarto & Yu, 2004; Mukhopadhyay et al., 2010). The higher expression of this protein in the albumen of the HFE hens suggests a higher defence of HFE albumen against bacterial invasion. The study of Bergsma et al (1985) reported that there are at least three cytoplasmic actin isotypes expressed in chicken non-muscle cells. Actin is a multiprotein complex in the cytoplasmic cytoskeleton which is involved in adenosine triphosphate (ATP) binding, DNA recombination and nucleosome mobilization, regulation of cell cycle, chromosome organization, DNA replication, DNA strand elongation, transcription DNA-templated. In response to DNA damage, actin also promotes homologous recombination (HR) repair including UV-damage excision and double-strand break by promoting nuclear actin polymerization, leading to drive motility of double-strand breaks. DNA damage either of one or both strands of the DNA helix are harmful and can have serious repercussions, both for the individual cells in which they occur and for the organism as a whole. In humans, actin protects mammalian eggs against chromosome segregation errors which can lead to miscarriages and occurrence of down syndrome (Mogessie & Schuh, 2017). In addition, the analysis using the Proteome discoverer identified some protein groups in the albumen from HFE hen eggs which are involved with various biological functions such as antimicrobial activity, structural and gelling characteristics, immune responses, protease inhibition, and metal ion binding or transport. Some of these protein groups are Vimentin, Histone H2B and 14-3-3 protein zeta, which were detected in the albumen of three out of 5 of the albumen from the HFE hen eggs, albeit absent in the LFE albumen group. Vimentin (VIM) is predominantly expressed in developing embryo and cells as a 57kDa class III intermediate

filament protein and has been reported to make stiff gel-like structures through polymerisation (Skalli & Goldman, 1991). It plays an important role in contributing to the mechanical integrity of the cell (Zehner et al., 1987; Sarria et al., 1994; Cancer Research, 2016). Histones are proteins that provides structural support to chromosomes and acts as antimicrobial peptides which kills bacteria, fungi, parasites and viruses, in chickens (Li et al., 2007) and a variety of animal hosts (Hoeksema et al., 2016). Taken together with the albumen height result in this study, the presence of these structural and antimicrobial proteins in the HFE albumen may play a significant role in influencing the bacteriostatic defence compared to the LFE hens.

The results of the Kirby–Bauer disc diffusion assay showed significant differences in the bacteriostatic properties of egg albumen of hens with varying feed efficiencies. The increased activity in the zones of inhibition observed in the HFE albumen suggests superior antibacterial effectiveness against gram-negative *Salmonella typhimurium* and gram-positive *Staphylococcus aureus*, compared to the egg albumen collected from LFE hens. Although there are no previous reports on the differences in the bacteriostatic properties of albumen in individual hens of varying feed efficiencies, previous work has indicated native egg white reduced the growth of gram positive and negative bacteria by about 20 – 31% and 29 – 31 % respectively (Abdel-Shafi et al., 2016). The images from the light microscope suggest a higher deformation of bacterial fat-insoluble cell walls from the HFE cohort, whose intact cells were lysed indicating higher mortality rates in both *Salmonella typhimurium* and *Staphylococcus aureus*. Lysozyme, an antimicrobial peptide which takes up about 3.4% of the total egg albumen peptides, targets bacterial cell membranes and is responsible for the disintegration of the lipid bilayer structure of gram positive bacteria cells (Jime'nez-Saiz et al., 2013). Ovomucin, which represents about 3.5% of the total egg albumen protein (Nakamura & Sato, 1964; Kovacs-Nolan et al., 2005; Omana et al., 2010) is configured as an interlinked network of gel-like fibres and responsible for the thickness and structure of the albumen as well as the

prevention of bacterial movement within the egg white (Strixner & Kulozik, 2011). Ovotransferrin, comprising 12%, starves the bacteria of iron (Legros et al., 2021), while the proteolytic activity of protease inhibitors also known as antiproteases counteracts the activities of bacterial enzymes (Guyot et al., 2013). Although there were comparable levels of major proteins in the albumen from both HFE and LFE hens, the differences observed in the bacteriostatic activity of the HFE albumen against *Salmonella* and *Staphylococcus* might be associated to the additional antimicrobial protein groups which were differentially expressed in the albumen from the HFE hens compared to that from the LFE hens.

5.6 Conclusion

This study investigated the differences in performance traits of ISA Brown hens in the late laying stage and explored conventional egg assessments as well as bacteriostatic and discovery proteomic approaches to identify the differences in the albumen of eggs collected from individual hens of varying feed efficiencies. It agrees with the hypothesis and concludes that hens retain their FCR rankings which they exhibited in early lay, up to at least 75 weeks with hens ranked as high feed efficient having a lighter body weight, a greater egg mass and consuming lesser feed. The eggs collected from the HFE hens have higher albumen height and Haugh unit compared to the hens ranked as LFE however eggs from LFE hens had higher yolk weights, widths, and pH values. The albumen from HFE hen eggs presented a greater bacteria inhibitory activity against *Salmonella typhimurium* and *Staphylococcus aureus*, compared to albumen from the LFE hens and this may be linked to the differences in proteome composition of the albumen. Since the quality of the egg once laid cannot be improved, efforts to attain and maintain a high albumen quality right from the production process should be amplified. The findings of the present study provide a basis for further studies to clarify the drivers of differences in albumen proteins as a result of variation in feed efficiencies of the hen. Research to explore the changes of antimicrobial peptides in the albumen as a result of variation in feed efficiency is merited.

CHAPTER SIX

6. Effect of restricted feeding on hen performance, egg quality and organ characteristics of individual laying hens

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This is the fifth result chapter of this thesis, and the objective was to evaluate the effect of temporary or permanent quantitative feed restriction beginning from point of lay, on performance traits, egg quality indices, organ characteristics and occurrences of fatty liver haemorrhagic syndrome of individually housed hens. In this intervention study, the hypothesis was that restricting feed intake to a maximum of 115 g per day from point of lay until 40 weeks (estimated as point when hens attain matured body weight), is a potential solution to control body weight and abdominal fat deposition, better feed to egg conversion efficiencies and improve the quality of the eggs.

6.1 Abstract

The objective of this study was to assess the impact on laying hen physiology, egg quality and organ characteristics, following permanent or temporary phases of restricted feeding. Two hundred and forty individually housed ISA Brown hens were monitored across two phases, in three treatments: *Ad libitum* feeding (ALF), temporary restricted feeding (TRF) and permanent restricted feeding (PRF), $n = 80$ hens per treatment. In Phase 1 (P1) - 22 to 40 weeks, the TRF and PRF hens were offered 115g of feed daily and in Phase 2 (P2) - 41 to 46 weeks, the TRF hens were transitioned to ALF status while the ALF and PRF hens remained as in P1. From 35 to 40 weeks, eggs were collected once weekly from 15 hens per treatment and assessed for differences in albumen, yolk, and shell variables. At 45 weeks, 10 hens each from the ALF and PRF groups were euthanized and differences in organ characteristics were assessed. In P1 average daily feed intake (ADFI), feed to egg conversion ratio (FCR) and body weight change (BWC) were higher ($p < 0.01$); EP and egg mass (EM) were lower ($p < 0.05$) while albumen height and Haugh unit were higher ($p < 0.01$) in hens that were allocated the TRF or PRF treatments compared to hens allocated the ALF treatment. In P2, TRF and ALF hens had a higher EP and EM than PRF ($p < 0.01$). BWC was highest in TRF and similar in both ALF and PRF, while ADFI and FCR were higher in TRF followed by ALF and least in the PRF treatment group ($p < 0.01$). ALF hens had a heavier abdominal fat pad and FHLS lesion score compared to PRF. Restricting hens to 115 g of feed per day from point of lay restrained body weight, improved FCR and albumen quality and reduced abdominal fat pad deposition and FLHS lesion scores in individually housed laying hens.

Key words: Restricted feeding; Albumen quality; FLHS; Abdominal fat; Laying hens; Body weight; Egg quality.

6.2 Introduction

Feed restriction is a management technique widely used in the poultry industry to control body weight, flock uniformity and performance (Lu et al., 2021; Scott et al., 1999), improve egg quality (Tolkamp et al., 2005), feed efficiency and profitability of bird flocks (Ewa et al., 2008; Olawumi, 2014) and manage disease (Han & Smyth, 1972). Quantitative measures such as reduced feed allowance offered multiple times a day (Taherkhani et al., 2010), skip-a-day feeding (Wilson et al., 2018), and time-restricted feeding (Saibaba et al., 2021), as well as qualitative methods where birds have access to different nutrient (protein, energy, or amino acids) allocations in diets (Ghazanfari et al., 2010) or feed dilution with low nutrient value ingredients (Rezaei & Hajati, 2009; Röhe et al., 2018), are established ways of restricting feed consumption in the poultry industry.

Egg weight (EW) and egg production (EP) are important measures of performance in the layer industry and appropriate feed supply is central to these outputs. However, research has shown that increased feed intake (FI) is not necessarily associated with greater EW (Anene et al., 2021) nor EP (Cerolini et al., 1994; Lacin et al., 2008) and the bigger EW sometimes seen in hens which consume more feed (Li et al., 2011) may be as a result of the increased deposition of excess dietary lipids in the yolks, thus leading to bigger yolk and bigger eggs. In egg producing flocks, increased body weight has been shown to be associated with poorer feed conversion ratio (FCR) and albumen quality (Lacin et al., 2008; Akter et al., 2018; Anene et al., 2021), reduced disease resistance (Han & Smyth, 1972), increased accumulation of abdominal fat, higher thermal temperatures (MacLeod and Hocking 1993; Oyedeji, Orheruata, and Omatsuli 2007), poorer bone health (Kolakshyapati et al., 2019) and the production of eggs with increased abnormalities thus making them unsellable (Ahsan-ul-haq et al., 1997; Anene et al., 2021).

In hens, sexual maturation and EP can only begin when a certain body weight threshold is reached (Renema et al., 1999), however, excessive increments in body weight (BW) starting early in life can lead to lifelong patterns that are detrimental for the hen and can result in poor production performance. Upon approaching mature BW, hens transition from a growth phase to a maintenance phase and heavier hens at this age have been shown to have poorer feed to egg conversion efficiency and prone to produce eggs with lower albumen quality (Anene et al., 2021). Therefore, the need to ensure a narrow variation in hen BW from an early age is just as important as attaining the right BW to trigger sexual maturation (Parkinson et al., 2015).

The consumption of feed above the nutrient and energy requirement for EP and body maintenance enhances the accumulation of abdominal fat, predisposing hens to heat stress (Simeneh, 2019), obesity and higher incidences of fatty liver haemorrhagic syndrome (FLHS) (Shini et al., 2019a). FLHS is a metabolic disorder triggered by fat infiltration into a structurally weakened liver (Couch, 1956). This disease is the reason for massive hepatic haemorrhage and death in caged hens and either in its acute or chronic forms resulting in a significant loss for egg producers (Schumann et al., 2003; A. Shini et al., 2019a). While feed control is one approach to manage BW variation in laying hens, the approach is problematic due to infrastructure requirements particularly in free range production systems and the risk of poor welfare due to satiety and feather pecking.

The effectiveness of permanent and temporary quantitative feed restriction from point of lay on hen performance, egg quality and organ characteristics of hens has not yet been extensively reported. The few documented studies that relate to feed restriction in hens are either dated (MacIntyre & Aitken, 1959; Gerry & Muir, 1976), have focused on other hens breeds (Ahsan-ul-haq et al., 1997; Moreira et al., 2012; Saibaba et al., 2021), were on a flock basis (Olawumi, 2014; Adewole et al., 2018), or in hens in the late stages of lay (Artdita et al., 2021). Our recent study has shown that natural appetite status attained from around point of lay is a stable trait

until at least 40 weeks of age (Anene et al., 2021), when hens reach their growth peak and mature bodyweight (ISA Brown, 2017). This suggests that the early life period is a critical window for establishing appetite throughout the egg cycle. Therefore, imposing a temporary lower FI status around the point of lay until mature body weight is reached may program their rate of consumption beyond peak growth point, when transitioned to *ad libitum* feed consumption. This would be of interest as an interim measure where the production system does not lend itself to permanent feed restriction. The objective of the study was therefore to evaluate the effectiveness of temporary or permanent quantitative feed restriction strategies to improve hens' performance, egg quality and organ characteristics traits in laying hens from point of lay until mid-lay stage.

6.3 Methodology

Animal ethics approval

The experiment was conducted at the Poultry Research Foundation (PRF) layer rearing facility, within The University of Sydney Camden Campus, New South Wales, Australia, in accordance with the 8th edition of the Australian code for the care and use of animals for scientific and experimental purposes (NHMRC, 2013). The procedures and activities conducted in this study, were reviewed, and approved by the University of Sydney Animal Ethics Committee.

Experimental animals and treatment groups

A total of 240 ISA Brown pullets were used in this study. The pullets were obtained from a certified breeder at 17 weeks of age and transported to the layer rearing facility. Each pullet was weighed and individually housed in 3-tier layer metal cages measuring 25 × 50 × 50 cm per cage. All pullets were then randomly assigned to one of three treatments: *ad libitum* (ALF), temporary restricted (TRF) and permanent restricted (PRF), $n = 80$ pullets per treatment. Hens were blocked based on initial BW to ensure the weight profile across the three treatment groups were similar. The experiment lasted for 24 weeks split into two phases starting from when the hens were 22 weeks of age, until 46 weeks of age. Phase 1 (P1) lasted for 18 weeks from 22 to 40 weeks of age, while Phase 2 (P2) lasted for 6 weeks from 41 to 46 weeks of age. The hens in the ALF treatment group were allowed unlimited access to feed throughout the day for both P1 and P2; in the PRF group, hens were offered 115 g of feed split in two feedings (morning and afternoon) for both P1 and P2 while birds in the TRF treatment group were offered 115 g of feed split in two feedings (morning and afternoon) in P1 and then transitioned to the ALF treatment status during P2.

Experimental diet.

A standard wheat-soy mash diet calculated to contain 16.3% crude protein and 2650 kcal/kg of gross energy was formulated by a commercial nutritionist according to (NRC, 1994) to meet

the nutritional requirements of the birds. The dietary composition, ingredients and nutrient composition of the diet offered to the hens are presented in Table 2.1. All birds were offered the same diet for the entire duration of the study and the diet was supplied in individual metal feeding troughs placed in front of the cages. The details of the feeding technique of the three treatments are as follows: For the ALF treatment, fresh diets were measured out weekly and offered freely to hens. In the PRF treatment group, a morning feed (40% of the 115 g breed recommended daily feed allocation) was offered first, followed by an afternoon feed (60% of the 115 g daily allocation) offered about 6 or 7 hours before the lights off moment. A similar feeding procedure was followed for the TRF treatment group, until P2 when they were transitioned to the ALF regimen. At the end of each week, feed not consumed was taken out, measured, and recorded. Hens were watered by automatic drinking nipples installed in the individual cages.

Hen Performance

The hens were allowed to acclimatise to the environment and attain peak egg production from 17 weeks until 22 weeks. Data collection for performance variables began at 22 weeks. Hens were individually weighed every four weeks from the start to end of the study period, using an electronic scale with a digital output. The difference in weight at the beginning and end of each phase was recorded as the body weight change (BWC) for that phase. Egg production (EP) was recorded daily and calculated in percentages (total number of eggs produced in seven days $\times 100$) / 7 (number of days of the week). Egg collection was done twice daily, and the weights were recorded per hen in grams using a digital weighing balance. The average daily feed intake (ADFI) was calculated (weekly feed offered – weekly feed unconsumed) / 7 (number of days in the week). Average egg mass (EM) per hen in grams was calculated (EP $\times$ EW / 100); where EP = Egg production, and EW = average egg weight, and the feed (to egg) conversion ratio (FCR) was calculated as the grams of feed consumed per gram of EM. All eggs collected during

the experimental period were assessed individually at point of collection for external abnormalities. The proportion of dirty, odd, frosty, blood stained, double yolked, shell-less, cracked, spotted and white shelled eggs from each hen was recorded. Egg quality analysis was carried out as described in Chapter 3.

Organ characteristics assessments.

At 45 weeks of age, 20 birds from the ALF and PRF treatment groups ($n = 10$ per treatment group) were randomly selected and euthanized by intravenous injection of sodium pentobarbitone in the wing vein. The keel bone was assessed and scored by the same observer, following modifications of protocols from (Wilkins et al., 2004; Scholz et al., 2008; Rufener et al., 2018). Keel bone fractures were assessed visually and by palpation of the intact carcass prior to necropsy and after removal of the skin. The differences noted were ranked on an ordinal scale of 0 to 3 (0 = no deformity, 1 = slight deformity, 2 = moderate deformity, 3 = severe deformity). Intra-abdominal fat (visceral fat) was excised using forceps, weighed with a digital balance and the abdominal fat weight as a percentage of BW was calculated. The liver was assessed for evidence for fatty liver haemorrhagic syndrome (FLHS) following the scoring system described by (Diaz et al., 1999) and compared with the pictorial images of haemorrhaged livers (Personal communication of Dr. Yeasmin Akter). Briefly, a liver score 0 indicates normal liver, liver score 1 indicates up to 10 subcapsular petechial haemorrhages, liver score 2 indicates more than 10 subcapsular petechial haemorrhages, liver score 3 – 5 indicates large and massive haemorrhages. The livers were then carefully removed as a whole organ and weighed. Histological, bacteriological, parasitological, or other laboratory examinations were not performed in this study. The gizzard, oviduct, proventriculus, pancreas and whole intestine were also excised, emptied where required, and weighed individually using an electronic balance. All organ weights were calculated as a percentage of BW, analysed, and compared across the treatment groups.

Statistical analysis

Data collected were analysed by one-way ANOVA using the PROC GLM procedure of SAS University Edition software (SAS Institute Inc., Cary, NC, USA) with feed intake group as the treatment factor. Differences among least squares means were computed using the pdiff statement in SAS. The individual hen within each feeding regimen group served as the experimental unit. Hens were blocked based on initial BW prior to the commencement of the study. All results are presented as least square means $\pm$ standard error of the mean (SEM). The p -value that denotes statistical significance was set at < 0.05 .

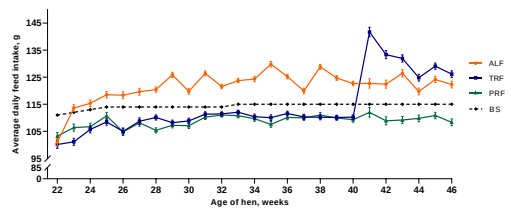
6.4 Results

Hen performance

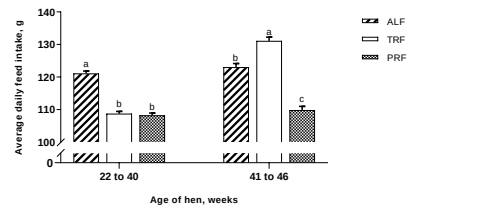
The weekly and overall hen performance of individual ISA Brown hens placed on different feeding regimens ($n = 80$ per feeding regimen group) for Phase 1 (P1) and Phase 2 (P2) are presented in Figure 6.1 and Supplementary Tables S1 – S6. In P1, the hens allocated the ALF treatment consumed more feed compared to those on the TRF and PRF ($p < 0.001$). The egg production was higher in the ALF treatment group compared to the PRF hens ($p < 0.01$). There was no significant difference in the egg weight across the three treatments, however, egg mass a product of the egg weight and egg production was slightly higher in the ALF hens compared to both TRF and PRF ($p < 0.05$). The FCR was higher (worse) in hens on the ALF treatment when compared with both TRF and PRF treatments ($p < 0.001$). The BW of all treatment groups was similar from 17 to 24 weeks and then became greater in the ALF hens from week 28 to 36 ($p < 0.001$). The body weight change (BWC) from 22 to 40 weeks was higher in ALF hens, compared to both TRF and PRF hens ($p < 0.01$).

In P2, the TRF hens had the highest feed intake, followed by the ALF hens while the PRF hens had the least feed consumption ($p < 0.001$). The egg production in the ALF and TRF hens were observed to be similar, and higher than in PRF hens ($p < 0.01$). The ALF and TRF hens were observed to have higher egg weight and egg mass than the PRF hens ($p < 0.05$), while the FCR became highest (worst) in the TRF hens, followed by the ALF hens and lastly in the PRF hens ($p < 0.001$). The BW was highest in the ALF hens compared to both the TRF and PRF hens, however, there was a higher BWC in the TRF hens compared to both ALF and PRF hens ($p < 0.01$).

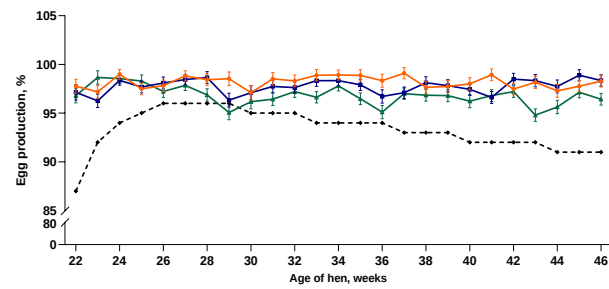
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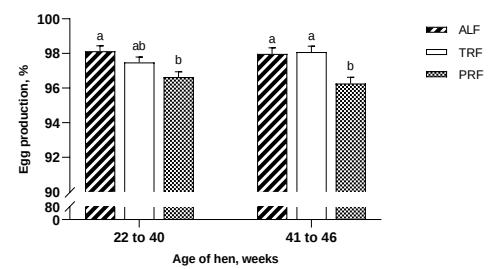
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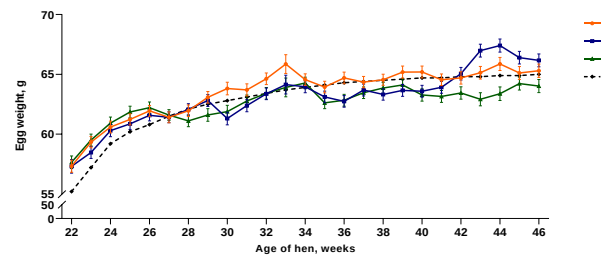
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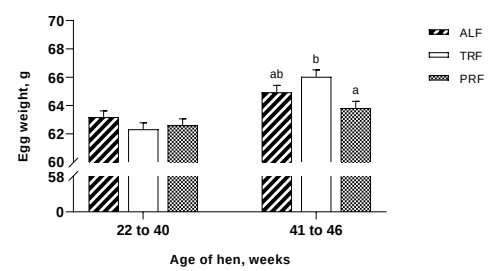
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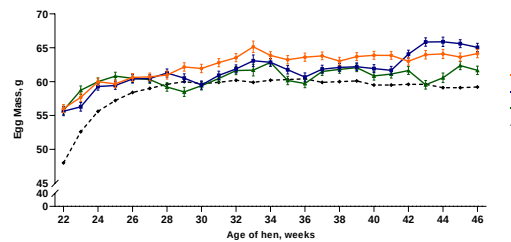
C1



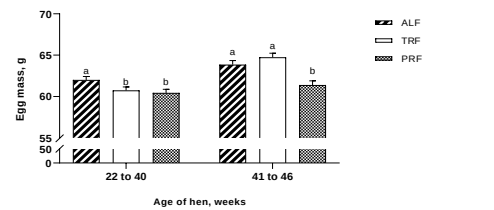
C2



D1

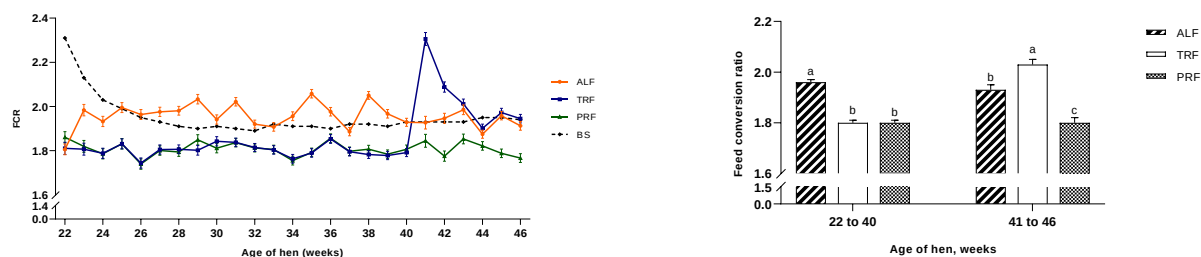
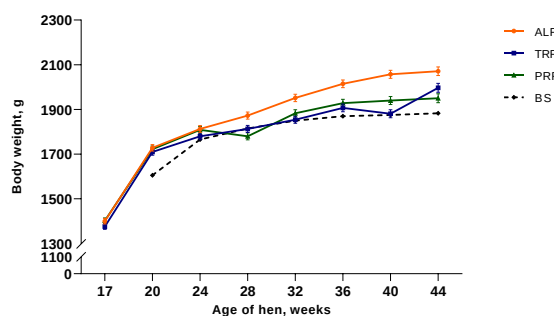
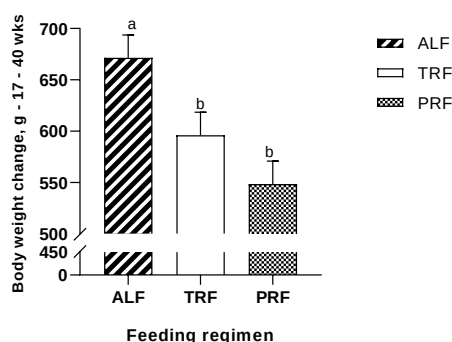
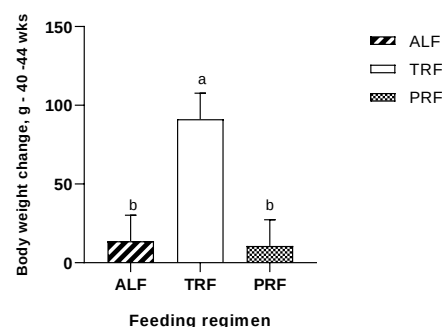


D2



E1

E2

**F****G1****G2**

ALF – *Ad libitum* feeding; TRF – Temporary restricted feeding; PRF – Permanent restricted feeding. a,b,c Means with different superscript indicate a significant difference between feeding treatment groups.

Figure 6.1 - The weekly and overall hen performance of individual ISA Brown hens placed on different feeding regimens (n = 80 per feeding regimen group) for Phase 1 (P1) and Phase 2 (P2). The weekly (22 to 46 weeks), Phase 1 (22 to 40 weeks of age) and Phase 2 (41 to 46 weeks of age) means of (A1; A2) average daily feed intake, (B1, B2) egg production, (C1, C2) egg weight, (D1, D2) egg mass and (E1, E2) feed to egg conversion ratio of individually housed ISA Brown hens on ALF, TRF and PRF feeding regimens, n = 80 hens per feed regimen group. The (F) weekly body weight of individual ISA Brown hens on ALF, TRF and PRF feeding

regimens from 17 to 44 weeks of age and (G1 and G2) body weight change in experimental hens on different feed regimens across different the two experimental phases.

The differences in the uniformity, total number of eggs laid, total amount of feed consumed, total egg mass and the FCR for the entire 24-weeks experimental period across the three feeding groups are presented in Table 6.1. There was no difference in the number of eggs produced across the three feeding treatments however, ALF hens consumed more feed and had an overall worse FCR, followed by the TRF and then the PRF ($p < 0.001$). The ALF and TRF hens had a higher egg mass compared to the PRF hens ($p = 0.02$) and the body weight uniformity was higher in the PRF hens.

Table 6.1 - Differences in clutch size, feed consumed, egg mass and feed conversion on an individual hen basis, of hens ranked into ad libitum (ALF), temporary restricted (TRF) or permanent restricted feeding (PRF) regimen from 22 weeks to 46 weeks of age

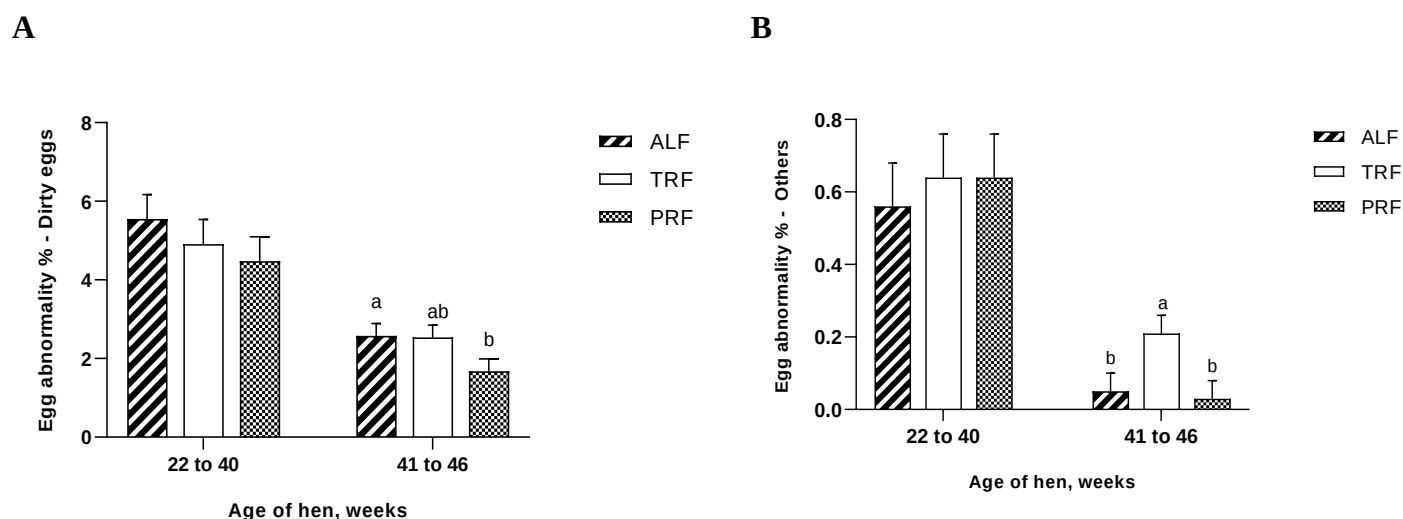
Feeding Regimen	Final body weight uniformity	Total count of eggs laid	Feed consumed (g)	Egg mass (g)	Feed conversion ratio
ALF	89.5	167	2892 ^a	1484 ^a	1.95 ^a
TRF	91.2	163	2651 ^b	1431 ^a	1.86 ^b
PRF	93.6	162	2511 ^c	1397 ^b	1.79 ^c
SEM	-	1.76	40.7	21.1	0.012
<i>p</i> -value	-	0.07	<0.001	0.02	<0.001

ALF – *Ad libitum* feeding; TRF – Temporary restricted feeding; PRF – Permanent restricted feeding. a,b,c – Means with different superscript indicate a significant difference between feeding treatment groups. SEM – Standard error of mean

Egg abnormality score

The frequencies of egg abnormalities across P1 and P2, are presented in Figure 6.2. In P1, there was no difference in the number of dirty eggs collected from all experimental groups. There was also no difference in the number of odd-shaped, frosty, blood stained, double-yolked, shell-less, cracked, spotted and white shelled eggs across the three treatments. In P2, the ALF hens produced more dirty eggs compared to the PRF hens ($p = 0.04$), and the TRF hens produced

more eggs with a combination of abnormalities which were either odd-shaped, frosty, blood stained, double-yolked, shell-less, cracked, spotted and white shelled compared to the hens in the ALF ($p = 0.03$) and PRF ($p = 0.01$) treatments.



ALF – *Ad libitum* feeding; TRF – Temporary restricted feeding; PRF – Permanent restricted feeding. a,b,c – Means with different superscript indicate a significant difference between feeding treatments. SEM – Standard error of meas. Others – Eggs consisting of abnormalities which include odd-shaped, frosty, blood stained, double-yolked, shell-less, cracked, spotted and white shelled.

Figure 6.2 - The percentage of (A) dirty eggs and (B) other egg abnormalities from individual ISA Brown hens placed on different feeding regimen of ALF, TRF and PRF across P1 (22 to 40 weeks) and P2 (41 to 46 weeks).

Egg quality

The albumen, yolk and shell characteristics of eggs from individual hens placed on different feed regimens between 35 to 40 weeks (during P1) are presented in Table 6.2. The PRF and TRF treatment hens had a higher albumen height ($p = 0.004$), Haugh unit ($p = 0.002$) and albumen index ($p = 0.002$) compared to the ALF hens. The albumen pH and yolk height were also higher in the PRF treatment hens ($p < 0.05$), compared to the ALF hens. However, albumen width and yolk weight were higher in the ALF hens ($p < 0.05$) compared to PRF groups. There was no difference in the yolk pH, egg weight, albumen weight, yolk colour and shell characteristics across the treatments.

Table 6.2 - Internal and external egg quality characteristics of eggs from individual ISA Brown hens aged between 35 to 40 weeks placed on ad libitum (ALF), temporary restricted (TRF) permanent restricted (PRF) feeding regimen.

Egg quality variables	Feeding regimens (n = 15 hens per feeding group)				
	ALF	TRF	PRF	SEM	P-value
Egg weight, g	64.5	64.6	63.3	0.60	0.29
Egg width, mm	44.7	50.1	44.2	3.19	0.36
Egg height, mm	56.8	57.1	56.5	0.19	0.08
Egg shape index %	78.7	87.6	78.3	5.46	0.40
Albumen height, mm	9.4 ^b	10.3 ^a	10.3 ^a	0.19	0.004
Haugh Unit	95.7 ^b	99.6 ^a	99.8 ^a	0.87	0.002
Albumen width, mm	66.9 ^a	65.1 ^b	64.8 ^b	0.59	0.02
Albumen index %	14.2 ^b	15.8 ^a	15.9 ^a	0.37	0.002
Albumen weight, g	39.5	40.1	39.3	0.44	0.42
Albumen weight %	61.3	62.1	62.0	0.28	0.12
Albumen pH	8.11 ^b	8.13 ^b	8.21 ^a	0.03	0.04
Yolk height, mm	16.4 ^b	16.7 ^a	16.6 ^a	0.06	0.004
Yolk width, mm	40.6	36.3	36.3	1.94	0.21
Yolk index %	42.4 ^b	45.9 ^a	45.8 ^a	1.01	0.02
Yolk weight, g	15.0 ^a	14.5 ^a	14.3 ^b	0.18	0.02
Yolk weight, %	23.3 ^a	22.5 ^b	22.6 ^b	0.24	0.04
Albumen:yolk ratio	2.6	2.8	2.8	0.04	0.05
Yolk colour	12.3	12.0	12.3	0.13	0.32
Yolk pH	5.87	5.89	5.89	0.01	0.18
Shell weight, g	6.8	6.7	6.6	0.07	0.21
Shell weight %	10.5	10.4	10.4	0.08	0.36
Shell thickness, mm	0.41	0.42	0.42	0.01	0.40
Shell breaking strength, g	4608	4848	4758	76.17	0.09

abc: Means within rows with different superscript are significantly different at the 5% level of significance. SEM – Standard error of mean
 ALF – *Ad libitum* feeding; TRF – Temporary restricted feeding; PRF – Permanent restricted feeding. Egg shape index = egg width/egg height
 $\times 100$; Albumen percentage = albumen weight/egg weight $\times 100$; Albumen index = Albumen height/albumen width $\times 100$; Yolk percentage

= Yolk weight/egg weight $\times$ 100; Yolk index = Yolk height/yolk width $\times$ 100; Yolk: Albumen ratio = Yolk weight/Albumen weight; Shell percentage = Shell weight/egg weight $\times$ 100.

Digestive organ characteristics

The digestive organ characteristics of 45-week-old hens from the ALF and PRF feeding treatments are presented in Table 6.3. At the time of slaughter, hens in the ALF feeding treatment were significantly heavier ($p < 0.001$) than hens on the PRF regimen. The absolute weight of the liver, intestines, gizzard, and oviduct was higher in the ALF treatment hens ($p < 0.05$) compared to the PRF hens. However, when adjusted to the body weight of the hen, there were no differences between the two treatments. The abdominal fat pad was heavier in the ALF hens both on an absolute basis ($p = 0.0002$) and when corrected to the body weight of the hen ($p = 0.001$). On the contrary, the weight of the proventriculus as a proportion of body weight was higher in the PRF hens compared to the ALF hens ($p = 0.004$).

Table 6.3 - Carcass characteristics of individual ISA Brown hens aged 45 placed on ad libitum (ALF) and permanent restricted feeding (PRF) regimen, n = 10 hens per feeding regimen group.

Carcass characteristic variables	ALF	PRF	SEM	P-value
Body weight, g – 45 weeks	2227 ^a	1947 ^b	37.91	<0.001
Abdominal Fat Pad, g	120.8 ^a	70.1 ^b	7.69	0.0002
Abdominal Fat Pad, % of BW	5.39 ^a	3.58 ^b	0.304	0.001
Liver weight, g	56.9 ^a	44.1 ^b	3.61	0.02
Liver weight, % of BW	2.53	2.27	0.144	0.22
Liver score	1.9 ^a	0.4 ^b	0.32	0.01
Intestinal weight, g	146.9 ^a	131.3 ^b	2.92	0.001
Intestinal weight, % of BW	6.63	6.76	0.21	0.65
Gizzard weight, g	44.2 ^a	34.8 ^b	1.48	0.0003
Gizzard weight, % of BW	1.98	1.79	0.07	0.07
Pancreas weight, g	3.9	3.6	0.46	0.66
Pancreas weight, % of BW	0.175	0.184	0.02	0.75
Oviduct weight, g	79.5 ^a	67.9 ^b	3.55	0.03
Oviduct weight, % of BW	3.57	3.49	0.16	0.77
Proventriculus weight, g	8.1	8.9	0.45	0.23
Proventriculus weight, % of BW	0.36 ^b	0.46 ^a	0.019	0.004
Keel bone score	1.4	1.1	0.29	0.48

ALF – *Ad libitum* feeding; TRF – Temporary restricted feeding; PRF – Permanent restricted feeding. abc: Means across rows with different superscript are significantly different at the 5% level of significance. SEM – Standard error of mean. FCR – Feed conversion ratio. ADFI – Average daily feed intake, BW – Body weight.

The pictograph template (Courtesy of Dr. Yeasmin Akter personal communication), used in assessing the livers from of the current study are presented in Figure 6.3, while the results of liver haemorrhage lesions from the hens in the current study are presented in Figure 6.4. The ALF treatment had more incidents of subcapsular petechial haemorrhages and thus a higher (poorer) liver score compared to the PRF hens ($p < 0.01$).

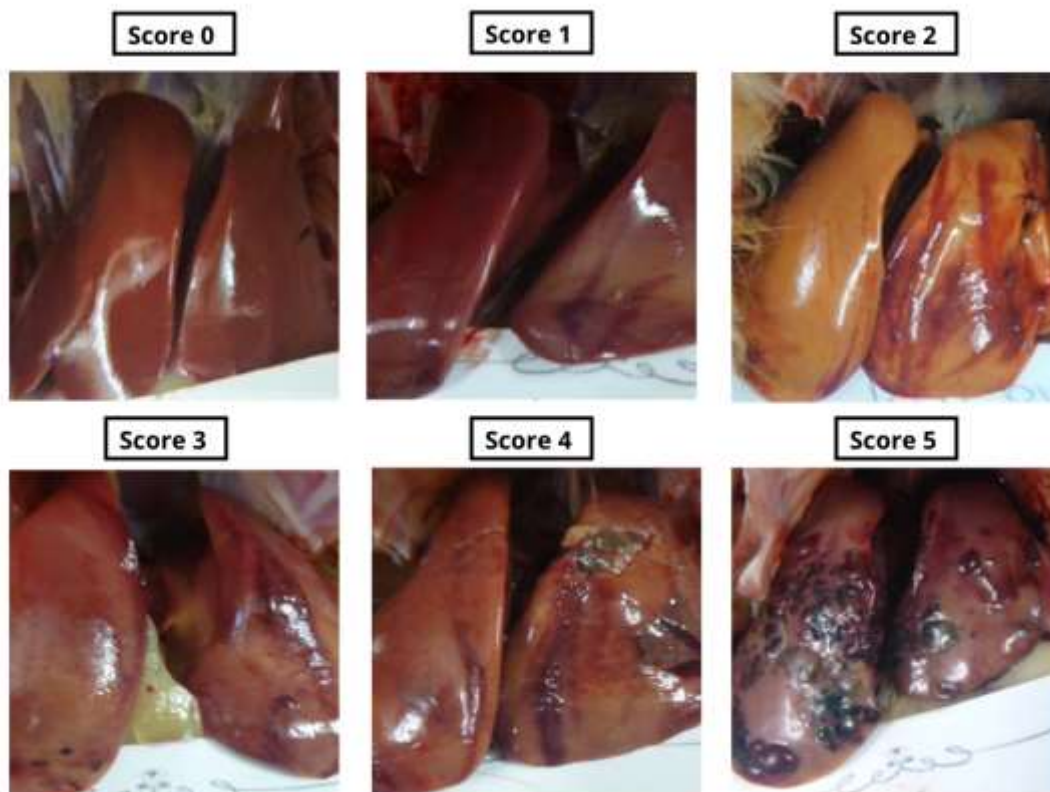
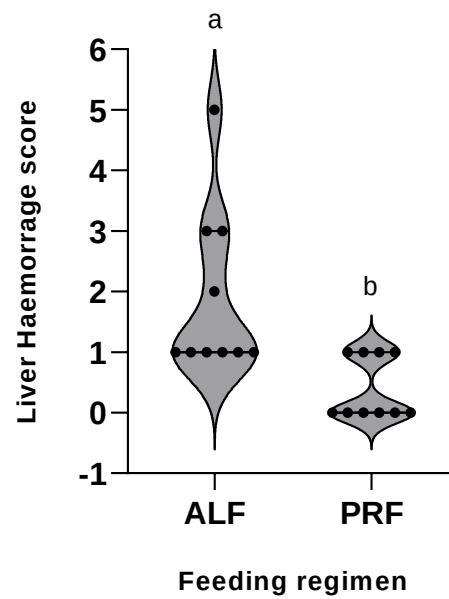


Figure 6.3 - Various levels of diagnosis of haemorrhage spectrum in laying hens. Courtesy of Dr. Yeasmin Akter'S personal communication. Liver score 0 indicates normal liver, liver score 1 indicates up to 10 subcapsular petechial haemorrhages; liver score 2 indicates more than 10 subcapsular petechial haemorrhages, liver score 3 – 5 indicates large and massive haemorrhages.



ALF – *Ad libitum* feeding; PRF – Permanent restricted feeding. abc: Means across rows with different superscript are significantly different at the 5% level of significance.

Figure 6.4 - The effect of feed restriction on liver haemorrhage lesions of individual ISA Brown hens aged 45 weeks placed on ad libitum (ALF) and permanent restricted (PRF) feeding regimen from point of lay.

6.5 Discussion

Variation in voluntary feed intake in hens can result in poor uniformity in body weight and may also negatively influence external and internal egg quality characteristics (Parkinson et al., 2015). In a previous study from our research group, it was reported that heavier hens in mid-lay stages had higher FCR and laid eggs with poorer albumen quality (Anene et al., 2021). The objective of this study was therefore to investigate a permanent and temporary quantitative feed restriction strategy, as an approach to control hen BW, improve FCR and egg quality. It further sought to better understand the effects of feed rationing and body weight on the incidences of FLHS and variation in other digestive organ characteristics of hens in the mid stages of lay.

In P1, the ALF hens consumed 12% more feed than the restricted fed hens. There was no difference in the overall egg weight of hens from the ALF vs PRF and TRF feeding treatments. The higher (worse) FCR observed in the ALF hens when compared with the PRF, is in agreement with studies that heavier hens have poorer feed efficiencies compared to lighter weight hens (Lacin et al., 2008; Anene et al., 2021), therefore more or heavier eggs will not be necessarily produced in response to greater feed consumption. As expected, by the conclusion of P1, as the hens attained growth maturity peak at 40 weeks of age, their body weights were seen to gradually stabilise, however, both TRF and PRF hens had a lower BWC and were lighter in BW, although still surpassing the breed target for that stage of lay (ISA Brown, 2017). The higher BWC seen in the ALF hens compared to the TRF and PRF hens, suggests that increased appetite and feed consumption in inefficient hens may be associated with an increase in body weight as excess nutrients and energy beyond what are required for the biological limits of egg production will be either excreted or used for BW gain and maintenance.

In P2, the spike in feed consumption, as well as the sharp BWC observed in the TRF hens suggests compensatory feeding behaviours when transitioned to *ad libitum* feeding status. It

also confirms that despite hens having attained mature bodyweight under a controlled feed intake regime, their *ad libitum* appetite was not restrained in line with previously imposed intake regime in P1 and thus highlights the limitations of the approach in the current study to control flock intake and BW from the point of lay period. The higher egg weights seen in the TRF hens in P2, could be because of the increased feed intake due to their transition from restricted to *ad libitum* feeding status. Excess nutrients not utilised for EP or body maintenance are eventually stored up as abdominal fat or synthesised to fat solids and deposited in the yolk which then contributes to bigger egg weights (Li et al. 2011). The higher egg production in the ALF hens in P1 and in ALF and TRF in P2, was contrary to the study where similar egg production was reported, irrespective of the differences in their feed consumption and BW (Anene et al., 2021). It was also different from the study by Ahsan-ul-haq et al (1997), who reported similar egg production in both *ad libitum* fed hens and hens fed 70% of feed requirement. Nonetheless, egg production across all three feeding treatments in both phases of current study exceeded the breed expectation for the ISA Brown breed between 22 to 46 weeks (ISA Brown, 2017). It is important to note that this high egg production may be because of the single housing arrangements of the birds in this study. Feed is the critical cost driver in egg production enterprises, and revenue in the egg industry is determined by feed intake, size, quality of the egg and the welfare and health condition of the hen. Although the PRF hens laid 5 less eggs in total for what period, they also consumed 380 grams less feed and had a similar egg size when compared with the ALF hens and had a lower (better) FCR compared to the ALF and TRF hens. The higher uniformity percentage of the PRF treatment is due to a narrow BW range and the lower uniformity percentage in the ALF treatment is linked to unrestrained BW gain. This study thus suggests that maintaining a feed consumption similar to the breed target, will improve flock uniformity and lead to positive economic effects for the egg producers. The higher number of dirty eggs observed in the ALF hens in P2 compared to the PRF, as well as

the higher number of other defects observed among the TRF hens in P2 in comparison with the PRF, is similar to the study of Saibaba et al (2021) and Anene et al (2021) and laying hens. It is also similar to the study by Heck et al (2004) where broiler breeder hens on an ALF regimen produced more defective eggs compared to their counterparts on a restricted feeding regimen. Eggs coated in faecal matter have a higher disposition to bacterial contamination and may be unsellable thus contributing to production losses for the egg producer.

The internal and external quality of the hen egg are important measures of performance in the poultry industry because of the safety and economic implications. The height of the egg albumen is an important indicator of egg freshness and quality and its corresponding Haugh unit (HU), a correction of albumen height with egg weight, is a standard industry measure of albumen quality. Both the height (thickness) of the albumen, influenced by the compatibility of structural peptides in the albumen, as well as the HU in P1 of the current study were seen to be higher in both the PRF and TRF hens compared to the ALF hens, suggesting that controlling feed intake and body weight has the potential to drive the production of eggs with greater albumen quality. A similar finding from Saibaba et al (2021), also reported significantly higher HU scores for eggs laid by time-restricted fed layers compared to control birds on an *ad libitum* feeding regimen. Previous studies have also shown that hens which consume lesser amount of feed and have a lower body weight produce eggs with higher albumen height and Haugh units compared to hens which consume more feed and have a heavier hens (Lacin et al., 2008; Anene et al., 2021). The albumen width was higher in the ALF hens and the resultant albumen index, a measure of the roundness of the albumen around the yolk was seen to be significantly worse in the ALF hens compared to the eggs from the PRF hens. The higher yolk height and resultant higher yolk index observed in the PRF hens compared to the ALF hens suggests a more round and compact yolk in eggs from feed restricted hens. In contrast, the bigger yolks seen in the ALF hens may be linked to the heavier weight of the ALF hens as excess fats not used by the

body for development maybe stored as abdominal fat or are deposited as fat solids in the yolks. This is similar to the finding from Akter et al (2018) who reported that 55-week old hens with a higher (poorer) FCR produced yolks with more saturated fatty acids compared hens with a lower (better) FCR. The colour of the yolk is an important trait of consumer preference which is largely influenced by the diet of the bird. The lack of difference in the yolk colour is noteworthy as it suggests that hens do not need to be fed beyond the breed suggestions and nutrient requirement to achieve a yolk colour which is acceptable by consumers. The lack of differences observed in the external characteristics of the egg suggests that hen feed intake beyond breed standard did not improve the egg shape index an important measure which relates to the ability of eggs to fit in boxes with fewer transit damages. An egg shell thickness of at least 0.33 mm has been estimated to be necessary for the eggs to have at least a 50% chance to withstand normal handling condition without breakage (Stadelman, 1986). The shell thickness recorded in the present study was similar across all three treatments and was at least 0.41 mm suggesting that restricting the hen feed intake to 115 g per day did not have any negative effect on the shell thickness and other shell quality characteristics. The eggshell breaking strength was not different between treatment groups indicating this variable was not negatively impacted by the feed restriction treatments.

Excessive body weight caused by increased feeding above the breed standard may present consequences on hens' health including excess abdominal fat deposition, increased internal core temperatures, obesity, a high incidence of lameness and high mortality due to skeletal disorders (MacLeod and Hocking 1993; Oyedele, Orheruata, and Omatsuli 2007). It may also contribute towards production of multiple yolks and oversized eggs, which are typically not marketable. In this study, a greater absolute and relative weight of the abdominal fat pad of the ALF hens was observed. To our knowledge, this has not been reported in laying hens, however, similar to the outcomes have been reported in investigations on breeder broilers (Robbins et

al., 1988; Renema et al., 1999). This highlights the negative implication of excess BW on the health and welfare of laying hens and emphasises the need to control and manage BW. The result from this study thus suggests that quantitative feed restriction measures is effective in reducing BW and abdominal fat pad deposition in laying hens. The hen oviduct is central to production performance and a disruption in its activity or pathology can affect egg quality and the profitability of commercial egg producers (Chousalkar & Roberts, 2008). Since there was no difference seen in the two treatments when corrected with the body weight of the hens, the bigger oviduct weight of the ALF hens observed in this study can be linked to the increased body weight. This outcome was also observed for the intestinal weight, and the weight of the gizzard. The 30% increase in the absolute liver weight observed in the ALF hen treatment, may also be linked to an increase in the yolk synthesis leading to larger egg weights of ALF hens similarly reported in (Akter et al., 2018). There was no difference in the keel bone score in the current study and this was similar to the findings from Kolakshyapati et al (2019) who observed no relationship between body weight and keel bone fractures and structures in their study with free-range hens.

FLHS is a metabolic disease that has been reported to be positively associated with body weight especially in caged hens (Schumann et al., 2003; Shini et al., 2019a). The study by Trott et al (2014) reports that approximately 97% of the FLHS affected birds were found to have large abdominal fat deposits, agreeing with earlier findings from King and Chen (1998), who reported that higher chances of FLHS is due to abnormal fat accumulation in the abdominal cavity, the visceral organs and liver cells of the chicken. The ALF hens in this study which were poorer feed to egg converters, consumed more feed and synthesised more energy than the requirements for egg production, resulting in a positive energy balance leading to greater body weight, higher abdominal fat deposition, were observed to have a higher FLHS lesion score compared to the PRF hens. The tendency of fatness in the ALF birds could be related to

fundamental metabolic differences in the partitioning of nutrients which influences negative biochemical and compositional changes on liver function, liver health, feed efficiencies and albumen quality.

6.6 Conclusion

The results of this study showed that hens demonstrate a compensatory feeding and weight gain pattern when transitioned from restricted to *ad libitum* feeding, thus restricting feed consumption until peak growth stage was reached was not effective in managing subsequent feed intake and controlling body weight gain. The comparison between ALF and PRF regimens shows that increased feed intake did not contribute towards heavier egg weights nor number of eggs laid. Transitioning hens from restricted feeding to *ad libitum* feeding at mature growth point did not control feed intake nor body weight. Although increased feed intake was seen to influence egg production and egg mass across the two phases, the benefits of PRF on feed to egg conversion ratio, internal egg quality, abdominal fat ratio and liver scoring may outweigh the higher egg rate obtained in the ALF hens. The findings from this study therefore concludes that in a controlled, individually caged experimental model, continued restriction of the feed intake of individual hens to a maximum of 115 g per day, split into two feeding slots from the point of lay, is an economic and biological strategy to control feed intake and body weight in ISA Brown hens. The outcome of this study may therefore provide suggestions to egg producers, on strategies to optimise productivity and profitability and inform the poultry industry on how controlling the feed consumption of hens will not only save production costs through better feed conversion efficiencies but will increase albumen quality and contribute to better hen welfare. However, continued feed restriction, controlled body weight, improved albumen quality and reduced abdominal fat deposition.

CHAPTER 7

7. General Discussion

Eggs, the major marketable product of hens, are economical protein sources with the ideal concentration of nutrients required by humans of all ages. The usefulness of eggs as a whole food has resulted in a high demand by its consumers, causing a corresponding supply from egg producers and thus, an increasing need to maintain both quantity and quality. Egg production, egg weight, feed intake, and body weight are key measures of productivity in the poultry industry. Uniformity in these traits can be linked to egg quality, egg safety and hen welfare and thus are a crucial consideration in ensuring optimum and sustainable production and profitability. Feed efficiency, FE, the rate of conversion of feed nutrients into egg mass is a performance trait of economic importance which has significantly improved over the years due to consistent genetic advancement, however, considerable variation still exists in FE among hybrid hens from the same genetic lines kept under common management and dietary conditions. Despite this background understanding, the extent of variation that exist, how early in lay these variations set in, the persistency of these variations and their effects on the quality and safety of the eggs across the hen laying period is not yet fully understood. The overarching objectives of this thesis was to investigate the inherent variation in hen performance traits of individual ISA Brown hens in early lay and evaluate its associations with egg quality characteristics and then rank hens based on the obtained feed efficiency and examine the production performance, egg quality, carcass characteristics trends. The hens in this study were explored across three laying stages: early lay, EL (25 to 30 weeks of age), mid lay, ML (35 to 40 weeks of age) and late lay, LL (70 to 75 weeks of age). As an intervention strategy, the secondary focus of this study was to investigate if controlling feed intake from point of lay will influence uniformity of other performance traits to drive improved hen performance, egg

quality indices and carcass characteristics. The hens in the intervention study were monitored across two phases: Phase 1, P1 (22 to 40 weeks of age) and Phase 2, P2 (41 to 46 weeks of age) under three treatments: *ad libitum* feeding (ALF), temporary restricted feeding (TRF) and permanent restricted feeding (PRF).

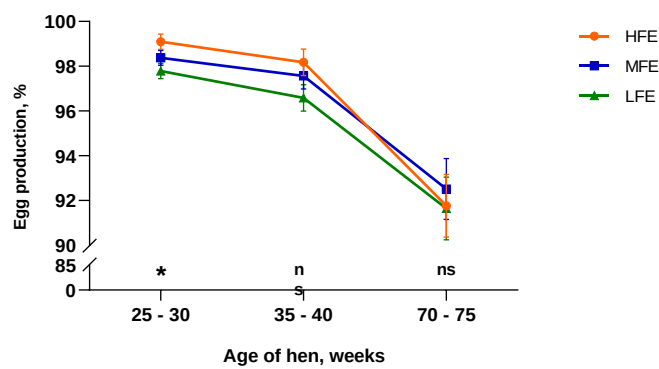
The findings from this research unveils the variation which exists in performance traits of individual hens, shows how these differences are attained from early lay and maintained until late laying stages, describes the influence of these variations on eggs quality and egg safety and contributes to the understanding of feed rationing in hen flocks as an intervention to control hen performance and improve egg quality. The following subheadings summarises the results contained in this thesis, their implications and suggests how these can be incorporated by egg producers, industry players and future researchers for increased and sustained productivity.

Hen performance

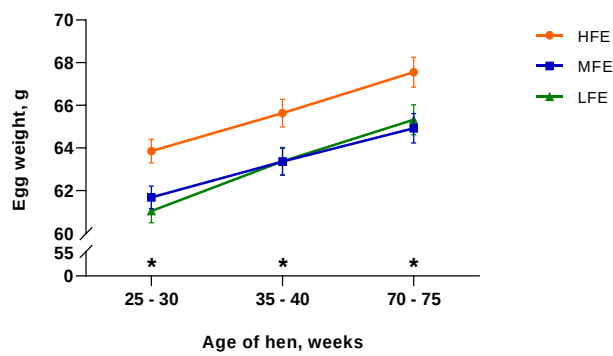
As presented in Figure 7.1, this research concludes that there are variations in hen performance traits which are present from early laying stages and persists throughout the entire laying period until at least 75 weeks. The hens which were heavier from EL remained so until the LL stages. Hens ranked as HFE had a lower appetite and consumed less feed compared to both MFE and LFE hens, a characteristic which they maintained until LL. The weight of the egg is of high producer and consumer preference and an important determinant of price. The HFE hens laid bigger eggs compared to the MFE and LFE hens all through the laying period. Differences in FE did not influence the rate of percentage egg production, except in the EL phase when HFE hens were seen to have a significantly higher rate of egg production. As expected, as hen age increased, egg weight got bigger and egg rate reduced over time. This study concludes that feed efficiency status established in early lay is a stable variable until at least 75 weeks of age and FI is the major driver of FCR and not EM. The continued positive association between hen

body weight and feed intake as well as between body weight and FCR in this study demonstrates that hens which consumed more feed and were heavier in weight, utilised feed nutrients less efficiently compared to lighter weight hens which had a lower feed intake.

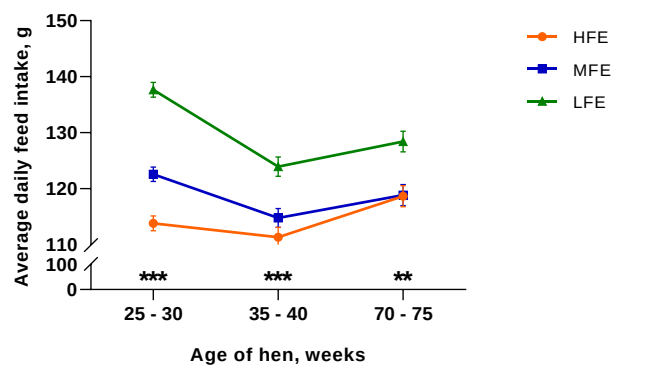
A



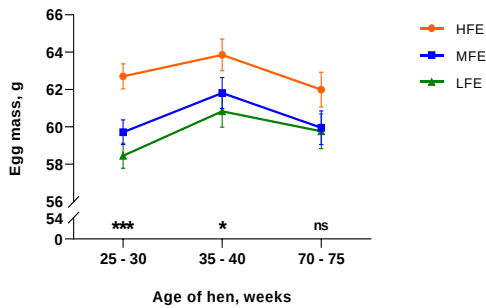
B



C

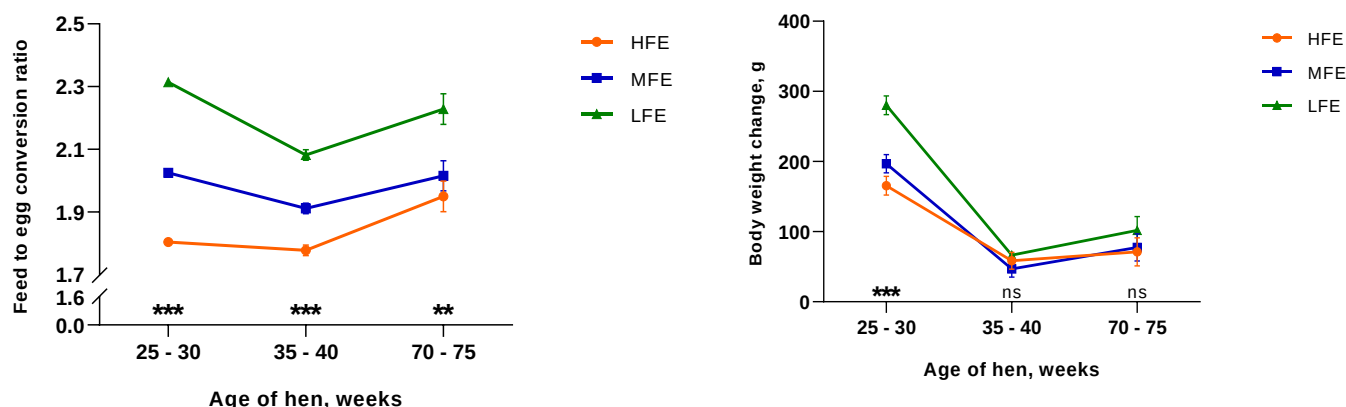


D



E

F



HFE – High feed efficiency; MFE – Medium feed efficiency; LFE – Low feed efficiency. * - $p = 0.05$; ** - $p = <0.01$; *** - $p = <0.0001$.

Figure 7.1 – The differences in (A) egg production (B) egg weight, (C) average daily feed intake, (D) egg mass (E) feed to egg conversion ratio and (F) body weight change of individually housed ISA Brown hens ranked as high feed efficiency (HFE), medium feed efficiency (MFE) and low feed efficient (LFE) $n = 50$ hens per FE group, across early lay (25 to 30 weeks of age) and mid lay (35 to 40 weeks of age) and late lay (70 to 75 weeks of age)

The findings from this research offers practical suggestions to egg producers on the importance of controlling feed intake and excessive body weight which influences feed to egg efficiency. This study suggests that controlling body weight and ensuring a narrow variation in hen body weight in flocks is just as important as attaining the right weight to initiate sexual maturation and this should be done as early in lay as possible because variations in body weight and other hen performance traits have been shown to set in early in lay and are persistent up till late lay. As an intervention, restricting feed intake to a maximum of 115g per day (according to the ISA Brown breed standard), split into two feeds a day, from the point of lay, reduced feed intake, controlled body weight and improved feed efficiency until at least 46 weeks of age. This study also recommends control of hen body weight to reduce abdominal fat deposition, fat content of the liver and incidences of FLHS and to improve the overall welfare of the hen. Since increased feed intake is not associated with increased egg numbers and egg weight, this study

informs the poultry industry on the importance of controlling the feed consumption of hens as this will not only save production costs through better feed conversion efficiencies but will reduce the incidences of abnormal eggs which leads to production losses. This study shows the relationship between excessive feed consumption and production of eggs with a reduced albumen quality, and thus recommends the control of feed intake in order to achieve egg production with higher albumen qualities and greater defence against microbial invasion.

It is important to note that this study was undertaken using individually housed animals in a highly controlled environment. This facilitated the measurement of relevant production traits on an individual basis which would typically be impossible to measure. However, this experimental model does not provide information on the impact of group dynamics that would be available using a flock set up. As the world moves towards phasing out caged housing systems, the findings reported in this study would benefit from further validation using flocks of hens and under non-caged housing conditions.

Age of first egg, weight of first egg and egg abnormality

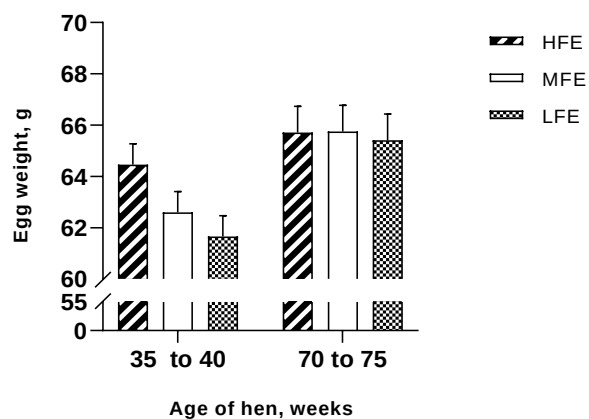
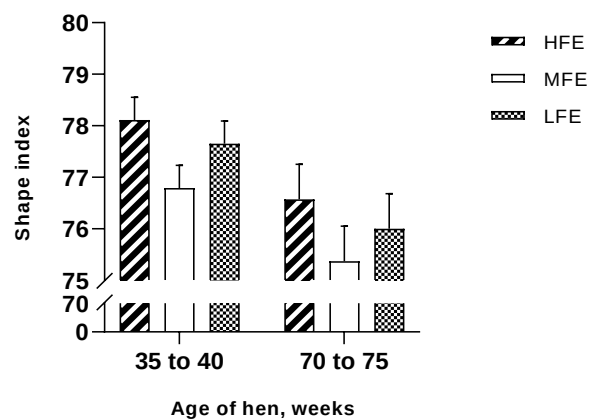
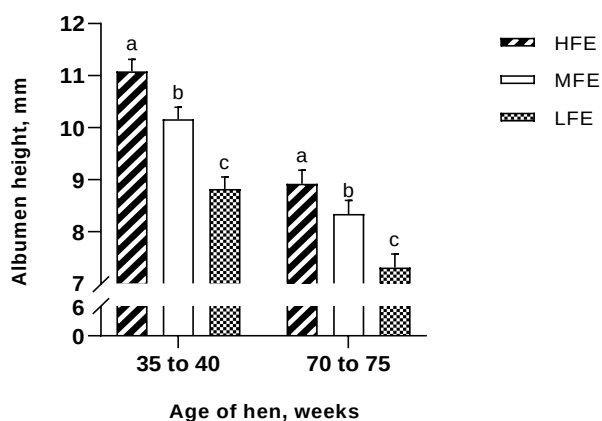
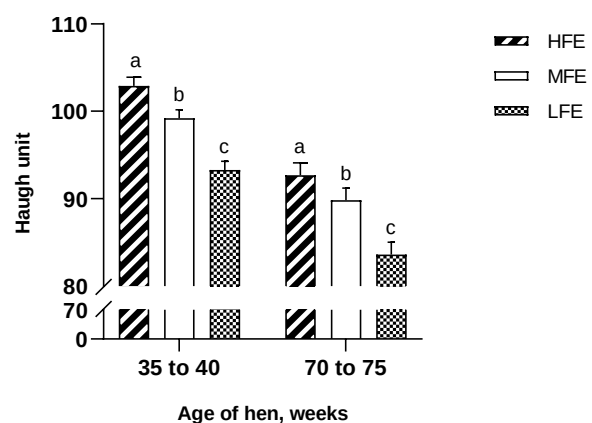
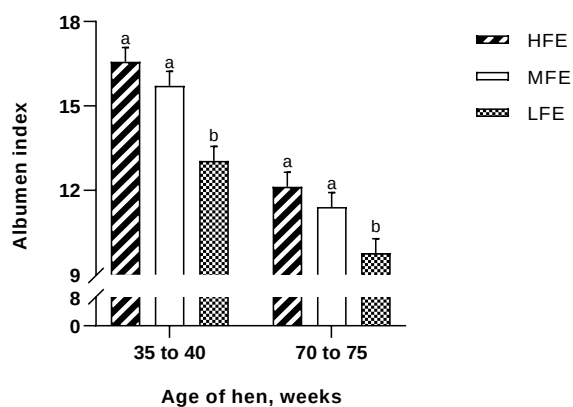
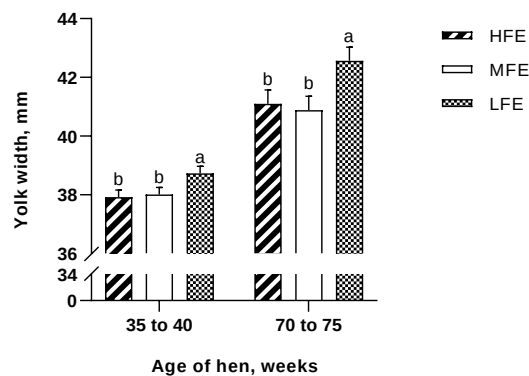
The age of the hen at first egg lay, has a significant effect on the weight of the first and subsequent eggs, where hens that begin to lay at an earlier age, tend to have a lower mean egg weight (Tserveni-Gousi, 1993). This research revealed that hens ranked as LFE attained sexual maturation six days earlier than HFE hens. In hens, ovulation is governed by body lipid /energy reserves and will not commence until a threshold is reached. The LFE hens likely reached this threshold sooner, due to greater bodyweight and therefore produced their first egg earlier than the HFE hens. Although the HFE hens came into sexual maturation later, their first egg was significantly heavier than the LFE hens first egg.

Egg abnormalities constitute an inefficiency for the egg industry. A higher incidence of egg abnormality was also observed among the LFE hens compared to the HFE hen groups in the ML. The differences in egg abnormalities formed in the early lay stage, between 25 to 30 weeks, continued into the mid lay stage, between 35 to 40 weeks, with eggs from the LFE group having more abnormalities compared to HFE hens. Dirty and soft-shelled eggs accounted for the prominent abnormalities observed, and eggs from the LFE cohort had 42% and 93% more dirty and soft-shelled eggs respectively, compared to the eggs from the HFE group. It is difficult to account for why LFE hens produced more dirty eggs, however, during the nutrient retention study, it was observed that LFE hens produced more faecal matter in line with greater feed consumption and this may be a contributory factor. The implications are that in addition to having poor FE, the hens from the LFE group also produced less saleable eggs. The feed restriction intervention study showed a higher occurrence of egg abnormality in the ALF hen group compared to the PRF treatment group. This confirms that controlling the feed intake of hens has a potential to reduce the occurrence and future probability of egg abnormality in hens.

Egg quality

As presented in Figure 7.2., the egg shape index which is the degree of roundness of the whole egg – an important characteristic in packaging and ensuring egg safety during transportation was not influenced by differences in FE, implying that that controlling the feed intake and resultant FCR does not affect the packaging safety of the egg. For internal egg qualities, the albumen height and Haugh unit was higher in the eggs from the HFE hens followed by MFE hens and the least in LFE hens. Albumen height and haugh unit are important measures of internal quality in the egg industry and this study thus suggests that hens ranked as HFE produced eggs with a higher internal quality. These variables were also seen to decrease as the

hens aged in agreement with previous studies that the albumen quality of the hen decreases as hen age increases. In the feed restriction study, the albumen height and HU was higher in the permanently restricted hen group compared to the ALF group. The albumen index, an indicator of roundness and even distribution of the albumen around the yolk was higher in the HFE hens and was higher in the eggs from PRF hens, implying that controlling feed intake in hens from point of lay, helped to improve the internal quality of eggs and provide greater protection against environmental shocks and defence against microbial invasion of the egg. The yolk weight and width were higher LFE hens, and this can be linked to increased feed intake, which results in a higher deposition of unused fat droplets into the yolks thus resulting in bigger yolks. Although a big yolk is desirable for consumers and for breeding purposes, an appropriate proportion to the albumen is important for increased defence against environmental shocks and microbial invasion. The eggshell provides structural integrity to the egg and is an important measure of quality of the egg and is largely influenced by nutrition. The lack of differences in the eggshell parameters irrespective of the three FE groups during the ML and LL stage, as well as in the three feeding regimen groups during the intervention study shows that controlling feed intake and maintaining reduced weight of hens does not affect the shell quality of eggs. However, it is important to ensure that the calcium intake in hens especially in late laying stages remain at the optimum levels to prevent compromise in the eggshell integrity.

A**B****C****D****E****F**

HFE – High feed efficiency; MFE – Medium feed efficiency; LFE – Low feed efficiency. a,b,c Means with different superscript indicate a significant difference between feeding groups.

Figure 7.2 - The means of (A) egg weight (B) shape index, (C) albumen height (D) Haugh unit and (E) albumen index (F) Yolk width of egg from individually housed ISA Brown hens ranked based on feed efficiency as HFE, MFE and LFE, n = 15 egg per FE group.

Nutrient digestibility, amino acids profile and organ characteristics

In this study, differences in feed efficiency status did not influence the nutrient retention capabilities of hens. However, the dry matter, nitrogen, and gross energy retention of the HFE hens was numerically higher than the LFE group. Although the LFE hens consumed more feed and hence dietary amino acids, there was lower concentrations of amino acids in the albumen of eggs from the LFE birds. This study therefore concludes that an increased efficiency of feed nutrients by the hens in the HFE group increases amino acid deposition in the egg albumen. The organ assessment of individually housed ISA Brown hens in this study concludes that at 45 weeks, hens with a higher feed to egg conversion ratio, which are heavier in weight, have a higher amount of abdominal fat pad deposition, even when corrected to the hen body weight. This trend was also seen in the intervention study, where hens on the ALF regimens presented a higher amount of abdominal fat in relation to their body weights. The positive correlations seen between feed intake and hen slaughter weight with liver fat content as well as a positive association between slaughter weight and FLHS lesion score suggests that increased feed consumption and body weight predisposes the hens to incidences of FLHS, and this was confirmed by the livers of LFE hens observed to have higher fat content and higher yellowness colour value, a higher FLHS lesion score had more swollen hepatocytes with distended cytoplasmic lipid vacuoles. In the intervention study, hens in the ALF group also had a higher FLHS lesion score suggesting that excessive feed intake and body weight predisposes hens to the occurrences of fatty liver haemorrhagic syndrome. In conclusion, disproportionate feed intake and body weight negatively influences the development of fatty and haemorrhaged livers

of hens, which may affect the hen's health and welfare. Thus, controlling feed intake at production farm sites may be useful to reduce the incidences of excessive abdominal fat deposition and fatty liver haemorrhagic syndrome in individual ISA Brown hens. For future works, it is suggested that organ characteristics studies should be conducted at unique time points such as early lay, mid lay and late laying stage, compared to this study where it was conducted only at 45 weeks of age (mid laying stage). This will be helpful in monitoring the onset and evolution of FLHS in laying hens.

Bacteriostatic properties and proteome profile

The Kirby-Bauer assay conducted in this thesis shows that albumen from HFE hens in LL had a stronger bacteriostatic effect against *Salmonella typhimurium* and *Staphylococcus aureus* than albumen from LFE hens. The study found 20 proteins, which were differentially expressed and unique to albumen from HFE hens but not detected in albumen from LFE hens. Some of these proteins had various structural and antibacterial properties and may play an important role in influencing albumen quality in the HFE group. Some important limitations experienced while conducting the proteomic analysis in this research was the use of limited number of albumen samples. For further research, it is recommended that albumen samples from more hens in the extreme categories be investigated for differences in the proteome profile. Secondly, during this study, albumen samples were collected when the hens were in the late stages of lay at 75 weeks old. For further research, it will be worthwhile to explore differences in the proteome profile of younger birds in order to monitor when differences are established and how they are maintained or diminished as the hens age.

Opportunities for future studies

As most countries of the world are leaning towards phasing out cages on egg farms, it will be worthwhile for new research and early career researchers to explore the variations in hen performance and how this influences egg quality in barns, free range or aviary farm settings. Previous work has established that the rearing stage is the most critical development stage of the layer life. It is worthwhile for more research to explore how feed intake and body weight of birds in rearing farms affects the hen performance and egg quality characteristics from 16 weeks until end of lay. Some research areas that can be further considered for investigation includes: Effect of body weight and feed efficiencies of individual hens on rate of follicle maturation and oviduct structure, and their relationships with egg quality, investigation on how temporary and permanent quantitative feed restriction influences hen performance, egg quality and carcass characteristics in the late lay of stages and detailed quantitative peptide tagged proteomic assessment of egg albumen from hens of varying body weights and feed efficiencies.

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Appendix

Appendix 1 - Weekly average daily feed intake (g) of individual ISA Brown hens aged between 22 to 46 weeks placed on *ad libitum* (ALF), Temporary restricted (TRF) and permanent restricted (PRF) feeding regimen, n = 80 hens per feeding regimen group.

Age of hen (weeks)	Feeding regimens			SEM	p-value	Breed standard
	ALF	TRF	PRF			
22	101.0	100.1	103.2	1.32	0.2451	111
23	113.6 ^a	101.1 ^b	106.4 ^c	1.29	<.0001	112
24	115.4 ^a	105.7 ^b	106.7 ^b	1.24	<.0001	113
25	118.5 ^a	108.4 ^b	110.7 ^b	1.21	<.0001	114
26	118.4 ^a	105.0 ^b	104.8 ^b	1.28	<.0001	114
27	119.6 ^a	108.7 ^b	108.1 ^b	1.25	<.0001	114
28	120.4 ^a	110.1 ^b	105.3 ^c	0.96	<.0001	114
29	125.9 ^a	108.2 ^b	107.2 ^b	0.98	<.0001	114
30	119.7 ^a	108.8 ^b	107.0 ^b	1.02	<.0001	114
31	126.4 ^a	111.3 ^b	110.2 ^b	0.88	<.0001	114
32	121.6 ^a	111.4 ^b	111.0 ^b	0.78	<.0001	114
33	123.7 ^a	112.0 ^b	110.8 ^b	0.77	<.0001	115
34	124.3 ^a	110.4 ^b	109.7 ^b	0.99	<.0001	115
35	129.8 ^a	110.0 ^b	107.6 ^b	1.05	<.0001	115
36	125.2 ^a	111.6 ^b	110.1 ^b	0.87	<.0001	115
37	119.9 ^a	110.3 ^b	110.0 ^b	1.00	<.0001	115
38	128.8 ^a	110.2 ^b	111.0 ^b	0.94	<.0001	115
39	124.7 ^a	110.1 ^b	109.9 ^b	0.86	<.0001	115
40	122.6 ^a	110.2 ^b	109.3 ^b	0.88	<.0001	115
41	122.7 ^a	141.7 ^b	112.0 ^c	1.73	<.0001	115
42	122.4 ^a	133.3 ^b	108.9 ^c	1.49	<.0001	115
43	126.4 ^a	131.9 ^b	109.2 ^c	1.34	<.0001	115
44	119.7 ^a	124.8 ^b	109.8 ^c	1.24	<.0001	115
45	124.2 ^a	129.1 ^b	110.8 ^c	1.15	<.0001	115
46	122.3 ^a	126.1 ^b	108.3 ^c	1.24	<.0001	115

a,b,c – Means with different superscript indicate a significant difference between feeding groups. SEM – Standard error of mean

Appendix 2 - Weekly rate of lay (%) of individual ISA Brown hens aged between 22 to 46 weeks placed on *ad libitum* (ALF), Temporary restricted (TRF) and permanent restricted (PRF) feeding regimen, n = 80 hens per feeding regimen group.

Age of hen (weeks)	Feeding regimens			SEM	p-value	Breed standard
	ALF	TRF	PRF			
22	97.8	97.1	96.8	0.75	0.63	87
23	97.2 ^{ab}	96.3 ^b	98.7 ^a	0.67	0.046	92
24	99.0	98.4	98.6	0.50	0.66	94
25	97.5	97.7	98.3	0.61	0.63	95
26	97.8	98.1	97.2	0.61	0.59	96
27	98.8	98.5	97.8	0.53	0.42	96
28	98.4	98.6	96.9	0.62	0.09	96
29	98.5 ^a	96.3 ^b	95.1 ^b	0.72	0.003	96
30	97.1	97.1	96.2	0.73	0.59	95
31	98.5	97.7	96.4	0.64	0.07	95
32	98.3	97.6	97.2	0.60	0.43	95
33	98.9 ^a	98.3 ^a	96.6 ^b	0.59	0.02	94
34	98.9	98.3	97.8	0.52	0.31	94
35	98.9 ^a	97.9 ^{ab}	96.5 ^b	0.59	0.02	94
36	98.3 ^a	96.7 ^{ab}	95.1 ^b	0.68	0.004	94
37	99.1 ^a	97.1 ^b	97.0 ^b	0.59	0.02	93
38	97.6	98.1	96.8	0.61	0.34	93
39	97.7	97.8	96.8	0.62	0.44	93
40	98.0	97.4	96.2	0.64	0.14	92
41	98.9 ^a	96.6 ^b	96.8 ^b	0.61	0.01	92
42	97.5	98.5	97.2	0.60	0.28	92
43	98.2 ^a	98.3 ^a	94.8 ^b	0.63	<.0001	92
44	97.3	97.7	95.6	0.67	0.07	91
45	97.8	98.9	97.1	0.57	0.10	91
46	98.3 ^a	98.4 ^a	96.4 ^b	0.60	0.04	91

a,b,c – Means with different superscript indicate a significant difference between feeding groups. SEM – Standard error of mean

Appendix 3 - Weekly egg weight (g) and of individual ISA Brown hens aged between 22 to 46 weeks placed on *ad libitum* (ALF), Temporary restricted (TRF) and permanent restricted (PRF) feeding regimen, n = 80 hens per feeding regimen group.

Age of hen (weeks)	Feeding regimens			SEM	p-value	Breed standard
	ALF	TRF	PRF			
22	57.3	57.3	57.6	0.53	0.88	55.2
23	59.3	58.5	59.5	0.49	0.29	57.2
24	60.6	60.3	60.9	0.50	0.68	59.2
25	61.2	60.9	61.8	0.47	0.34	60.2
26	61.9	61.6	62.2	0.47	0.63	60.8
27	61.4	61.4	61.6	0.46	0.95	61.5
28	62.0	62.1	61.1	0.48	0.31	62.1
29	63.1	62.8	61.6	0.51	0.10	62.5
30	63.8 ^a	61.3 ^b	61.9 ^b	0.53	0.002	62.8
31	63.7	62.4	62.8	0.51	0.18	63.1
32	64.6	63.4	63.4	0.49	0.12	63.4
33	65.9	64.1	63.9	0.79	0.16	63.7
34	64.6	64.0	64.3	0.49	0.69	63.9
35	63.9	63.1	62.6	0.49	0.15	64.1
36	64.7 ^a	62.7 ^b	62.8 ^b	0.49	0.01	64.3
37	64.4	63.7	63.5	0.49	0.40	64.4
38	64.5	63.3	63.9	0.47	0.19	64.5
39	65.2	63.7	64.1	0.50	0.09	64.6
40	65.2 ^a	63.6 ^b	63.3 ^b	0.50	0.02	64.7
41	64.5	63.9	63.2	0.49	0.13	64.7
42	64.7	65.0	63.4	0.52	0.08	64.8
43	65.1 ^b	67.0 ^a	62.9 ^c	0.54	<.0001	64.8
44	65.9 ^b	67.4 ^a	63.4 ^c	0.55	<.0001	64.9
45	65.1 ^{ab}	66.4 ^a	64.2 ^b	0.54	0.02	64.9
46	65.3 ^{ab}	66.3 ^a	64.0 ^b	0.55	0.02	65

a,b,c – Means with different superscript indicate a significant difference between feeding groups. SEM – Standard error of mean

Appendix 4 - Weekly egg mass (g) of individual ISA Brown hens aged between 22 to 46 weeks placed on *ad libitum* (ALF), Temporary restricted (TRF) and permanent restricted (PRF) feeding regimen, n = 80 hens per feeding regimen group.

Age of hen (weeks)	Feeding regimens			SEM	p-value	Breed standard
	ALF	TRF	PRF			
22	56.0	55.6	55.8	0.61	0.90	48.0
23	57.7 ^{ab}	56.3 ^b	58.7 ^a	0.63	0.03	52.6
24	60.0	59.3	60.0	0.54	0.56	55.6
25	59.7	59.4	60.8	0.57	0.21	57.2
26	60.6	60.4	60.5	0.61	0.96	58.4
27	60.7	60.5	60.3	0.57	0.88	59.0
28	61.0 ^a	61.3 ^a	59.2 ^b	0.61	0.04	59.6
29	62.2 ^a	60.5 ^a	58.5 ^b	0.65	0.001	60.0
30	61.9 ^a	59.5 ^b	59.4 ^b	0.62	0.005	59.7
31	62.8 ^a	60.9 ^b	60.5 ^b	0.61	0.02	59.9
32	63.5	61.9	61.6	0.61	0.06	60.2
33	65.1 ^a	63.1 ^{ab}	61.7 ^b	0.86	0.02	59.9
34	63.9	62.9	62.8	0.57	0.35	60.2
35	63.2 ^a	61.7 ^{ab}	60.2 ^b	0.62	0.003	60.3
36	63.6 ^a	60.7 ^b	59.7 ^b	0.62	<.0001	60.4
37	63.8 ^a	61.8 ^b	61.5 ^b	0.58	0.01	59.9
38	63.0	62.1	61.8	0.58	0.29	60.0
39	63.7	62.2	62.1	0.59	0.10	60.1
40	63.9 ^a	61.9 ^b	60.8 ^b	0.61	0.002	59.5
41	63.9 ^a	61.7 ^b	61.1 ^b	0.59	0.002	59.5
42	63.0 ^{ab}	64.1 ^a	61.6 ^b	0.59	0.02	59.6
43	64.0 ^b	65.8 ^a	59.5 ^c	0.63	<.0001	59.6
44	64.1 ^a	65.9 ^a	60.6 ^b	0.68	<.0001	59.1
45	63.6 ^a	65.6 ^b	62.4 ^a	0.60	0.001	59.1
46	64.1 ^a	65.1 ^a	61.7 ^b	0.62	0.0004	59.2

a,b,c – Means with different superscript indicate a significant difference between feeding groups. SEM – Standard error of mean

Appendix 5 - Weekly feed to egg conversion ratios of individual ISA Brown hens aged between 22 to 46 weeks placed on *ad libitum* (ALF), Temporary restricted (TRF) and permanent restricted (PRF) feeding regimen, n = 80 hens per feeding regimen group.

Age of hen (weeks)	Feeding regimens			SEM	p-value	Breed standard
	ALF	TRF	PRF			
22	1.81	1.81	1.86	0.025	0.25	2.31
23	1.98 ^a	1.81 ^b	1.82 ^b	0.026	<.0001	2.13
24	1.93 ^a	1.79 ^b	1.79 ^b	0.023	<.0001	2.03
25	1.99 ^a	1.83 ^b	1.83 ^b	0.023	<.0001	1.99
26	1.96 ^a	1.74 ^b	1.74 ^b	0.023	<.0001	1.95
27	1.98 ^a	1.80 ^b	1.80 ^b	0.021	<.0001	1.93
28	1.98 ^a	1.81 ^b	1.79 ^b	0.020	<.0001	1.91
29	2.03 ^a	1.80 ^b	1.85 ^b	0.022	<.0001	1.90
30	1.94 ^a	1.84 ^b	1.81 ^b	0.021	<.0001	1.91
31	2.02 ^a	1.84 ^b	1.84 ^b	0.019	<.0001	1.90
32	1.92 ^a	1.81 ^b	1.81 ^b	0.018	<.0001	1.89
33	1.91 ^a	1.80 ^b	1.81 ^b	0.020	0.0001	1.92
34	1.96 ^a	1.76 ^b	1.76 ^b	0.020	<.0001	1.91
35	2.06 ^a	1.79 ^b	1.79 ^b	0.019	<.0001	1.91
36	1.98 ^a	1.85 ^b	1.86 ^b	0.020	<.0001	1.90
37	1.89 ^a	1.80 ^b	1.80 ^b	0.019	0.001	1.92
38	2.05 ^a	1.78 ^b	1.81 ^b	0.018	<.0001	1.92
39	1.97 ^a	1.78 ^b	1.78 ^b	0.019	<.0001	1.91
40	1.93 ^a	1.79 ^b	1.81 ^b	0.019	<.0001	1.93
41	1.93 ^b	2.31 ^a	1.84 ^c	0.029	<.0001	1.93
42	1.95 ^b	2.09 ^a	1.78 ^c	0.023	<.0001	1.93
43	1.99 ^a	2.01 ^a	1.85 ^b	0.022	<.0001	1.93
44	1.87 ^a	1.90 ^a	1.82 ^b	0.019	0.011	1.95
45	1.96 ^a	1.97 ^a	1.79 ^c	0.019	<.0001	1.95
46	1.91 ^a	1.94 ^a	1.77 ^c	0.020	<.0001	1.94

a, b, c – Means with different superscript show a significant difference between feeding groups. SEM – Standard error of mean

Appendix 6 - Weekly body weight of individual ISA Brown hens aged between 22 to 46 weeks placed on *ad libitum* (ALF), Temporary restricted (TRF) and permanent restricted (PRF) feeding regimen, n = 80 hens per feeding regimen group.

Age of hen (weeks)	Feeding regimens			SEM	p-value	Breed standard
	ALF	TRF	PRF			
17	1400	1376	1402	12.39	0.26	-
20	1728	1709	1722	14.85	0.67	1605
24	1812	1780	1809	15.27	0.25	1765
28	1872 ^a	1813 ^b	1780 ^b	15.92	0.0002	1815
32	1951 ^a	1854 ^b	1883 ^b	16.36	0.0001	1850
36	2015 ^a	1907 ^b	1928 ^b	17.48	<.0001	1870
40	2057 ^a	1881 ^c	1940 ^b	18.23	<.0001	1875
44	2071 ^a	1997 ^b	1950 ^b	22.40	0.0001	1883

a, b, c – Means with different superscript show a significant difference between feeding groups. SEM – Standard error of mean