EFFECTS OF DIETARY CALCIUM, PHYTOESTROGEN RICH DIETS AND ESTROGEN ON INTESTINAL CALCIUM TRANSPORT PROTEINS, EGG AND EGGSHELL QUALITY IN MATURING LAYER HENS.

Ali Asghar Saki

(B.Sc. Univ. of Razi, M.Sc. Univ. of Tarbiate-Modaress, Tehran, Iran)

A THESIS SUBMITTED TO THE UNIVERSITY OF ADELAIDE

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

DEPARTMENT OF ANIMAL SCIENCE
THE UNIVERSITY OF ADELAIDE
WAITE CAMPUS GLEN OSMOND 5064
AUSTRALIA

SEPTEMBER 1998

Corrigenda

General:
Text marked with an *, reader should refer to corrigenda for changed text or explanation of existing text. Where possible, changes have been made on the text directly.

Tegel pullets used in these studies were of a Rhode Island Red x Australope cross and not Red Island x Australope or Red - Island Red x Australope has indicated in the text.

Summary:

P.xv L5 "in laying cycle" should read "in the laying cycle"
P.xv L8 "enzyme the" should read "the enzyme"
P.xvi L1 change "affect" to "affected"
P.xvi L16 insert "with respect to" before "egg shell quality"
P.xvii L5 "to switch" should read "in switching"
P.xviii L8 change "group" to "groups"
Pxix L5 change "effect" to "affect"

Chapter 1:
Introduction:

P3 L17 - 21 "financial losses, with these reductions, annual global" should read "financial losses. However, annual global..."
P3 L21 change "however" to "therefore".
P4 L13 change "a doubt of" to "is doubt concerning".
P4 L16 change "effect" to "affect".
P4 L22 - 23 change "effect on egg" to "affect egg".

Literature review:

P5 L2 change "peivitelline" to "perivetilline".
P5 L11 delete "to".
P6 L4 delete "After the egg formation,..." sentence is unnecessary.
P6 L12 delete "on".
P8 L8 change "These fibres of restrict" to "These fibres restrict".
P10 L8 delete "are".
P10 L9 changes "layers" to "layer".
P10 L14 change "is" to "in".
P10 L18&21 change "surface of crystal layer" to "surface crystal layer".
P12 L14 change "increase" to "increased".
P12 L21 "metabolism, is now thought" should read "metabolism, it is now thought".
P13 L2,8&9 change "effect", "effects" and "effected" to "affect", "affects" and "affected".
P14 L18 change "while" to "While".
P15 L1-3 "Differences in the sysnthesis..." deleted. Statement is unnecessary.
P15 L6 change "affect" to "effect".
P15 L8,9&12 change "receptors", "as a ligand.." and "acids" to "receptor", "as ligand.." and "acid", respectively.
P16 L4 change "to binding in" to "binding to".
P18 L2 "stores and can" should read "stores can".

P19	L2	Add the phrase: "All these studies were conducted using mammals (human and rats)."
P19	L6	"could effect on" should read "could affect".
P19	L13	change "increase" to "increased".
P21	L12	"limited ability of laying hens to retention" should read "limited ability of older laying hens to retain".
P22	L4-7	"National Research Council (1994)…." Is to be ignored.
P25	L3	delete "an"
P25	L5	"hens can be reduced" should read "hens which can be reduced"
P26	L19	"compartments play specific functions in nutrients" should read "compartments has specific functions in nutrient".
P27	L13	delete " with in…..cells".
P29	L6-7	"The membranes with two distinct parts, produce a polarised cell (cell with two opposite sides) and tight junctions combine.." should read "The membranes with two distinct sides, separated by tight junctions combine.."
P30	L8	delete duplicate "in the".
P32	L21	change "responsive" to response".
P32	L22	insert "acid" after "arachidonic".
P33	L10	change "effect" to "affect"
P43	L6	"involved calcium" should read "involved in the calcium"
P43	L20	"which stimulated" should read "which was stimulated"
P44	L1	change "effect" to "affect"
P51	L14-17	To read "Although the production of the vitamin D3 metabolite (1,25(OH)2D3) is increased by the indirect effect of estrogen on production of 1-hydroxylase enzyme in the kidney, it is not known whether estrogen directly effects calcium transport across the small intestine in pre-laying hens.
P52	L7	"may play specific" should read "which may play a specific".
P52	L14	change "point-lay" to "point-of-lay".

Chapter 2:

P56	L4&13	change "effect" for "affect".
P56	L12	"and made free" should read" and were kept free".

Chapter 3:

P70	L21	insert "tissue" after "jejunal".
P71	L16	"entrocyte" should read "enterocyte".
P75	L13	"reduced remarkably" should read "tended to be reduced".
P76	L7	"nuclear" should read "nuclei".
P76	L11	"shown" should read "indicates".

Chapter 4:

P80	L19	"In addition, …" should read "In addition, the Tegel is a strain used in many poultry farms in Australia".
P90	L23	"lead to reduce of protein" should read "lead to a reduced protein"
P91	L2-5	read should note that these trends were not statistically different.
P96	L6-7	White Leghorn (Hy-Line) and Black Tegel (Hi-sex) were the commercial breeds.

Chapter 5:

P116 L4 "due to strain" indicates a possible interaction between estrogen and type of bird.

Chapter 6:

P134 Fig 6.1 Title should read "Plasma concentration of phytoestrogens in leghorn pullets maintained on either meat meal or soybean diets"

P146 L4 Oviduct weight and shell gland weight tended to increase but these differences were not significant. Refer to Table 6.3, P132.

Chapter 7:

P160 Table 7.4 Title should read "Soybean and Phytoestrogen intake in 22 week old Leghorn pullets"

Chapter 8:

P184 L12-14 Should read "Also, the expression of calbindin D_{28K} has be observed in the cytoplasm and nuclei of small intestinal enterocytes (Lawson, 1978)."

P189 L8-10 Should read "High levels of total phytoestrogen, diadzein and geinstein are observed in plasma of those hens fed soybean compared with meat meal, this is due to the negligible levels of the phytoestrogens found in meat meal based diets."

References:

P205 R4 Qin and Klandorf (1995) the journal is "Comparative Biochemistry and Physiology".

P207 R3 Simkiss (1961) the journal is "Biological Reviews".

TABLE OF CONTENTS

TABLE OF CONTENTS II

LIST OF TABLES X

LIST OF FIGURES XII

LIST OF PLATE XIV

SUMMARY XV

DECLARATION XX

DEDICATION XXI

ACKNOWLEDGMENT XXII

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION 2

1.2. LITERATURE REVIEW 5

1.2.1. EGG FORMATION 5

1.2.2. STRUCTURE AND FUNCTION OF EGG SHELL 7

1.2.2.1. The macrostructure and function of egg shell 7

1.2.2.2. The microstructure and function of egg shell 8

1.2.4. STRUCTURE AND FUNCTION OF ESTROGEN RECEPTORS 15

1.2.5. CALCIUM FUNCTION, CALCIUM DIETARY, REQUIREMENT AND HOMEOSTASIS IN PRE-LAYING HENS 16

1.2.5.1. Calcium function in pre- laying hens 16

1.2.5.2 Dietary calcium supplementation 18

1.2.5.3 Calcium Requirement 19

1.2.5.4 Calcium homeostasis in pre-laying hens 23

1.2.6. REGULATION OF INTESTINAL NUTRIENT TRANSPORT........ 26

1.2.6.1. Intestinal structure and function 27

1.2.6.2. Calcium transport in the small intestine 30

1.2.6.3. Transcellular calcium transport 32

1.2.6.4. Paracellular calcium transport........ 37

1.2.6.5. Regulated calcium transport in intestine and shell gland........ 38

1.2.6.6. Structure and function of calcium binding protein. 39

1.2.6.7. Intestinal enzyme activity 43

1.2.6.8. Role of hormones 44

1.2.7. ESTROGENIC DIETS (PHYTOESTROGENS)........ 48

1.2.8. AIM AND OBJECTIVES 51

CHAPTER TWO: GENERAL MATERIALS AND METHODS

2.1. INTRODUCTION 54

2.2. ANIMALS 54

2.3. GROWTH ASSESSMENT 54

2.4. FEED AND FEED ANALYSIS 55

2.4.1. DRY MATTER MEASUREMENT 55

2.4.2. ENERGY MEASUREMENT 55

2.4.3. PROTEIN MEASUREMENTS 55

2.4.4. FIBRE AND FAT CONTENT OF FEED........ 56

2.4.5. MINERALS 56

2.4.6. PHYTOESTROGEN IDENTIFICATION AND MEASUREMENT........ 56

2.4.7. FEED INTAKE AND FEED CONVERSION RATIO (FCR) ... 57

2.4.8. NUTRIENT RETENTION ... 57

2.5. EGG PRODUCTION ... 58

2.5.1. EGG AND EGG SHELL CHARACTERISTICS ... 58

2.6. SAMPLING OF SMALL INTESTINE ... 59

2.7. INTESTINAL MORPHOMETRY ... 60

2.8. IMMUNOHISTOCHEMICAL METHODS ... 63

2.8.1. ESTROGEN RECEPTOR LOCALISATION IN JEJUNUM TISSUE ... 63

2.8.2. CALCIUM BINDING PROTEIN LOCALISATION IN THE JEJUNUM TISSUE ... 65

2.9. JEJUNUM HOMOGENATE AND ENZYMES ACTIVITIES ... 65

2.9.1. PROTEIN DETERMINATION ... 65

2.9.2. ALKALINE PHOSPHATASE ASSAY ... 65

2.9.3. CA, MG, CA-MG ATPASE ASSAY ... 66

2.9.4. NA/K ATPASE ASSAY ... 68

CHAPTER THREE: SMALL INTESTINAL EXPRESSION OF CALCIUM BINDING PROTEIN (CALBINDIN D_{28K}) AND ESTROGEN RECEPTORS IN 15 AND 26 WEEK OLD ISA-BROWN HENS

3.1. INTRODUCTION ... 70

3.2. MATERIALS AND METHODS ... 71

3.3. RESULTS ... 71

3.3.1. LOCALISATION OF CALCIUM BINDING PROTEIN (CALBINDIN D_{28K}) AND ESTROGEN RECEPTOR 71

3.3.2. INTESTINAL MUCOSA MORPHOMETRY 75

3.3.3. ENZYME ACTIVITY IN JEJUNAL HOMOGENATE 75

3.4. DISCUSSION 76

CHAPTER FOUR: INTESTINAL FUNCTION AND REPRODUCTIVE CAPACITY OF 18-22 WEEK OLD TEGEL PULLETS IN RESPONSE TO ADMINISTRATION OF EXOGENOUS ESTROGEN

4.1. INTRODUCTION 79

4.2. MATERIALS AND METHODS 81

4.3. RESULTS 82

4.3.1. FEED EFFICIENCY AND GROWTH 82

4.3.2. NUTRIENTS RETENTION 83

4.3.3. EGG AND EGG SHELL CHARACTERISTICS 84

4.3.4. INTESTINAL MUCOSA MORPHOMETRY 85

4.3.5. ENZYMES ACTIVITIES OF JEJUNAL HOMOGENATE 86

4.3.6. CALCIUM BINDING PROTEIN (CALBINDIN D_{28K}) EXPRESSION 87

4.3.7. DISCUSSION 90

CHAPTER FIVE: COMPARISON BETWEEN 18-22 WEEK OLD LEGHORN AND TEGEL PULLETS FOR INTESTINAL FUNCTION AND REPRODUCTIVE CAPACITY IN RESPONSE TO EXOGENOUS ESTROGEN AND DIETARY CALCIUM.

5.1. INTRODUCTION **95**

5.2. MATERIALS AND METHODS **96**

5.3 RESULTS **97**

5.3.1 GROWTH AND FEED INTAKE 97

5.3.2 NUTRIENT RETENTION 100

5.3.3. EGG AND EGGSHELL CHARACTERISTICS 103

5.3.3.1. Egg characteristics *103*

5.3.3.2. Egg shell characteristics *104*

5.3.4. THE INTESTINAL MUCOSA MORPHOMETRY 108

5.3.5. ENZYME ACTIVITY 110

5.3.6. CALCIUM BINDING PROTEIN LOCALISATION 110

5.4. DISCUSSION **116**

5.4.1. GROWTH AND FEED EFFICIENCY 116

5.4.2. NUTRIENT RETENTION 117

5.4.3. EGG AND EGGSHELL QUALITY 118

5.4.4. INTESTINE MORPHOMETRY 120

5.4.5. ENZYME ACTIVITY 121

5.4.6. EXPRESSION OF CALCIUM BINDING PROTEIN (CALBINDIN D_{28K}) 122

5.4.7. CONCLUSION 122

CHAPTER SIX: INTESTINAL FUNCTION OF 10-14 WEEK OLD LEGHORN PULLETS IN RESPONSE TO EXOGENOUS ESTROGEN AND A HIGH PHYTOESTROGEN DIET

6.1. INTRODUCTION 126

6.2. MATERIALS AND METHODS 128

6.3. RESULTS 130

6.3.1. GROWTH AND FEED EFFICIENCY 130

6.3.2. GROWTH AND CAPACITY OF THE GASTROINTESTINAL TRACT AND OVIDUCT 130

6.3.3. PHYTOESTROGEN INTAKE AND PLASMA PHYTOESTROGEN LEVELS 133

6.3.4. NUTRIENTS RETENTION 134

6.3.5. INTESTINAL MUCOSAL MORPHOLOGY 135

6.3.6. ENZYME ACTIVITY 138

6.3.7. EXPRESSION OF CALCIUM BINDING PROTEIN (CALBINDIN D_{28K}) 140

6.4. DISCUSSION 143

6.4.1. GROWTH AND FEED EFFICIENCY 143

6.4.2. GROWTH OF THE GASTROINTESTINAL TRACT AND OVIDUCT 144

6.4.3. PHYTOESTROGEN INTAKE AND PLASMA PHYTOESTROGEN LEVELS 145

6.4.4. NUTRIENT RETENTION 145

6.4.5. INTESTINAL MUCOSAL MORPHOLOGY 147

6.4.6. INTESTINAL ENZYME ACTIVITY 148

6.4.7. EXPRESSION OF CALCIUM BINDING PROTEIN IN THE JEJUNAL TISSUE 149

6.4.8. CONCLUSION 150

CHAPTER SEVEN: INTESTINAL FUNCTION AND REPRODUCTIVE CAPACITY OF 18-22 WEEK OLD LEGHORN PULLETS IN RESPONSE TO EXOGENOUS ESTROGEN AND HIGH PHYTOESTROGEN DIET

7.1. INTRODUCTION 153

7.2. MATERIALS AND METHODS 155

7.2.1. PHYTOESTROGEN IDENTIFICATION AND MEASUREMENT 156

7.3. RESULTS 157

7.3.1. GROWTH AND FEED EFFICIENCY 157

7.3.2. GROWTH OF GASTROINTESTINAL TRACT AND OVIDUCT 157

7.3.3. PHYTOESTROGEN INTAKE AND PLASMA PHYTOESTROGEN LEVELS 160

7.3.4. NUTRIENT RETENTION 161

7.3.5. EGG AND EGG SHELL CHARACTERISTICS 163

7.3.6. INTESTINAL MUCOSAL MORPHOLOGY 165

7.3.7. INTESTINAL ENZYME ACTIVITY 165

7.3.8. EXPRESSION OF CALCIUM BINDING PROTEIN 169

7.4. DISCUSSION 172

7.4.1. GROWTH AND FEED EFFICIENCY 172

7.4.2. GROWTH OF THE GASTROINTESTINAL TRACT AND OVIDUCT 172

7.4.3. PHYTOESTROGEN INTAKE AND PLASMA PHYTOESTROGEN LEVELS 173

7.4.4. NUTRIENT RETENTION 174

7.4.5. EGG AND EGG SHELL CHARACTERISTICS 174

7.4.6. INTESTINAL MORPHOLOGY 175

7.4.7. INTESTINAL ENZYME ACTIVITY 176

7.4.8. EXPRESSION OF CALCIUM BINDING PROTEIN (CALBINDIN D_{28K}) 178

7.4.9. CONCLUSION 178

CHAPTER EIGHT: GENERAL DISCUSSION

GENERAL DISCUSSION 182

REFERENCES 192

APPENDIX: PUBLICATIONS 211

LIST OF TABLES

Table 1.1: The composition of eggs from various species of poultry 8

Table 3.1: Intestinal mucosa structure in 15 and 26 week old Isa- brown hens 75

Table 4.1: The effect of estrogen on growth and feed intake in 18-22 week old Tegel pullets.......... 83

Table 4.2: Egg and egg shell characteristics response to estrogen levels in 18-22 week old Tegel pullets 85

Table 4.3: Intestinal mucosa structure in 22 week old Tegel pullets in response to estrogen levels 86

Table 5.1: The effect of calcium, strain and different level of estrogen on body characteristics and daily feed intake 99

Table 5.2: Nutrient retention in response to calcium, strain of pullets and estrogen levels 101

Table 5.3: Calcium, strain and different levels of estrogen on egg and eggshell characteristics 106

Table 5.4: Intestinal morphometry regarding calcium, strain and estrogen levels.......... 109

Table 5.5: Enzyme activities in homogenate of Jejunum from 22 week old Leghorn and Tegel pullets, response to interaction of estrogen and calcium........ 115

Table 6.1: Diets and different levels of estrogen (treatments) 128

Table 6.2: Feed intake and growth in 10-14 week old Leghorn pullets in, response to different diets or treated with different levels of estrogen 131

Table 6.3: Gastrointestinal tract weight, capacity and oviduct weight (g/100g body wt.) in response to different diets or treated with different levels of estrogen in 14 week old Leghorn pullets 132

Table 6.4: Soybean and phytoestrogens intake in 10-14 week old Leghorn pullets.. 133

Table 6.5: The retention of various nutrients by 10-14 week old Leghorn pullets fed different diets or treated with different levels of estrogen 135

Table 6.6: Intestinal mucosal morphometry in 14 week old Leghorn pullets fed different diets or treated with different levels of estrogen 137

Table 7.1: Diets and different levels of estrogen (treatments) 155

Table 7.2: Feed intake and growth, in response to different diets or treated with different levels of estrogen in 18-22 week old Leghorn pullets 158

Table 7.3: Gastrointestinal tract weight, capacity and oviduct weight (g/100g body wt.) in response to different diets or different levels of estrogen in 22 week old Leghorn pullets 159

Table 7.4: Soybean and phytoestrogens intake in 14 week old Leghorn pullets........ 160

Table 7.5: The retention of various nutrients by 22 week old Leghorn pullets fed different diets or treated with different levels of estrogen 162

Table 7.6: Egg and egg shell characteristics of 18-22 week old Leghorn pullets fed different diets or treated with different levels of estrogen 164

Table 7.7: Intestinal mucosal morphometry in 22 week old Leghorn pullets fed different diets or treated with different levels of estrogen 167

LIST OF FIGURES

Figure 1.1 a, b: The physical components of egg and egg shell 9

Figure1.2(a, b): The dimensions and geometry of hen's egg 11

Figure 1.3: Process of calcium requirement in laying hen 20

Figure 1.4: Illustrated calcium homeostasis in laying hens 25

Figure 1.5 (a, b): Cell division and migration in the epithelium of small intestine mucosa 28

Figure.1.6: Potential pathways and mechanism for calcium transport across intestinal epithelium 39

Figure 1.7. The EF-hand Ca^{2+}-binding protein with two α-helices, loop and calcium which is coordinated by seven oxygen ligands 40

Figure 1.8: Structure of the major classes of phytoestrogen (flavonoinds), position of hydroxy group on A and B rings are listed for selected examples within each class 50

Figure 3.1: (a) Alkaline phosphatase activity in jejunal homogenate at 15 and 26 week old Isa-brown hens; (b), ATPase enzyme activity in jejunal homogenate at 15 and 26 week old Isa-brown hens 76

Figure 4.1 Nutrient retentions in response to different levels of estrogen.. ... 84

Figure 4.2: (a) Alkaline phosphatase activity of jejunal homogenate in 22 week old Tegel pullets; (b) ATPase activities of jejunal homogenate in 22 week old Tegel pullets 87

Figure 5.1: The effect of calcium and estrogen interaction on the retention of nutrients 102

Figure 5.2: Interaction of different levels of calcium and estrogen on egg and eggshell characteristics 107

Figure 6.1: Plasma concentration of phytoestrogens in maintained on either meat meal or soybean diets 134

Figure 6.2: Alkaline phosphatase (A) and ATPase enzyme (B), activities in response to meat meal and soybean meal diets, respectively; Alkaline phosphatase (C) and ATPase enzyme (D), activities in response to different levels of estragen (E_2, µg/ kg body weight/day) respectively 139

Figure 7.1: Plasma concentration of phytoestrogens in 22 week old Leghorn pullets maintained on either meat meal or soybean diets 161

Figure 7.2: Alkaline phosphatase (A) and ATPase enzyme (B) activities in response to meat meal and soybean meal diets; Alkaline phosphatase (C) and ATPase (D), enzyme activities in response to different levels of estrogen 168

LIST OF PLATE

Plate 2.1: Typical Haematoxylin and Eosin stained sections of duodenal, jejunal and ileal tissue taken from pre-lay hens 61

Plate 3.1: Immunohistochemical localisation of estrogen receptors (A) and Calbindin D_{28K} (B) expression in typical jejunum sections from 15 and 26 week old Isa Brown hens 72

Plate 4.1: Immunohistochemical localisation of Calbindin D_{28K} expression in typical section from 22 week old Tegel pullets treated with exogenous estrogen 88

Plate 5.1: Immunohistochemical localisation of calbindin D_{28K} in crypt (1) and villus (2) regions of typical jejunal tissue taken from 22 week old Leghorn (A) and Tegel (B) pullets in response to low (2%) or high (4%) dietary calcium and given intramuscular inject of either 0 or 50 μg estrogen/ kg body weight/day 112

Plate 6.1: Immunohistochemical localisation of calbindin D_{28K} in crypt (1) and villus (2) regions of typical jejunal tissue taken from 14 week old Leghorn pullets in response to cereal and soybean meal diets and different levels of exogenous estrogen 141

Plate7.1: Immunohistochemical localisation of calbindin D_{28K} in crypt (1) and villus (2) regions of typical jejunal tissue taken from 22 week old Leghorn pullets in response to cereal and soybean meal diets and different levels of exogenous estrogen 170

SUMMARY

It is well established that the calcium requirement of laying hens increases dramatically during sexual maturation and the onset of lay. Indeed laying hens require two grams of calcium to form the shell of each 60 g egg. This high demand for calcium is affected by vitamin D_3 $(1,25(OH)_2D_3)$ during the process of calcium uptake in laying cycle. The uptake of this Ca by the small intestine is modulated by hormonal changes of the birds during sexual maturation. It has been found that the production of vitamin D_3 $(1,25(OH)_2D_3)$, a known modulator of calcium metabolism in the kidney is regulated by 1-hydroxylase enzyme the which is affected by estrogen (E_2). However the possibility of a direct effect of estrogen on calcium transport at the intestinal level has not been adequately studied. The question investigated in this study, was whether there is a possibility that estrogen can modulate the proteins associated with calcium transport, (e.g. calcium binding protein, calbindin D_{28k}) in the intestinal tissue during sexual maturation.

The first study reported was designed to determine the temporal and spatial expression of estrogen receptors (ER) and calcium binding protein in jejunal tissue. Results demonstrated that the localisation of estrogen receptors preceded CaBP (calbindin D_{28k}) expression. These findings indicate the potential of estrogen to induce calbindin D_{28k} in the intestinal tissue. Therefore, it may be concluded that estrogen may increase calcium absorption and consequently calcium retention. It was also found that age had no significant effect on ATPase (Ca, Mg and Ca/Mg) enzyme activity. In contrast, alkaline phosphatase activity increased with age, this corresponded with egg production and shell formation at this particular stage. Mucosal structure, except for

duodenum surface area, was not affect* by age.

In the second study, Tegel pullets (a common strain in Australian poultry farms) were tested to determine the effect of different levels of exogenous estrogen on calcium retention and egg and eggshell characteristics. Similar results were found with respect to different treatments on feed intake, weight gain, feed conversion ratio and intestinal morphometry. At 10 µg /kg body weight /day, E_2 increased shell thickness and shell weight per cm^2 when compared with the other treatments, (0 and 100 µg E_2/kg body weight/day), but calcium retention was reduced by this level (10µg) of estrogen. No significant effect was observed on either ATPase or alkaline phosphatase activity, or the expression of calbindin D_{28k} in the jejunal tissue. These results may be a special case peculiar to this strain of pullets

The information gathered led to a third study which aimed at determining the effect of different strains of pullets (Leghorn and Tegel) and various levels of exogenous estrogen (0, 10, 50 and 100 µg/kg body weight/day) in hens fed either low or high (2% and 4%) calcium diets. The results showed Leghorn pullets were more responsive to 50 µg of estrogen and high level of calcium (4%) in* egg shell quality, although the calcium retention and enzyme activity in this strain were lower than in Tegel pullets. Feed intake was significantly greater ($P<0.01$) in Tegel than Leghorns. In contrast, feed conversion and shell gland weight were significantly lower ($P<0.05$) and ($P<0.001$) respectively, in the Tegels compared with Leghorns. Jejunal crypt depth was greater in birds maintained on a high compared with a low level of calcium in the diet. Ileum villus surface area showed the same trend in Tegel pullets as in Leghorns. No differences were recognised in calbindin $_{D28k}$ expression in this study. These

findings led to a new hypothesis in regard to the response of Leghorn pullets to phytoestrogen, which can be found in commercial poultry diets, when the legume, soybean, is included as a source of protein as it contains considerable phytoestrogen.

Two studies (in 10- and 18- week old Leghorn pullets) were conducted to examine the effects of exogenous estrogen and phytoestrogen to *switch on the expression of calbindin D_{28k} and consequently change the calcium pool in bone before the onset of lay (10-14 weeks of age). Also their effects were tested on calcium retention and egg and eggshell characteristics at the onset of lay (18-22 weeks of age).

First, 10-14 weeks-old pullets with 50 μg of estrogen was chosen as a treatment since it had an effect on shell quality in the previous study (experiment 3) and Leghorns were used as they showed greater response to 50 μg estrogen. Soybean meal was used as a replacement for meat meal as a source of protein in the cereal diets and the two dietary treatments were used at two estrogen levels (0 and 50 μg). The results indicated that weight gain and efficiency of feed conversion were significantly reduced ($P<0.001$) by soybean meal compared with meat meal. In contrast, weight and the capacity of the small intestine were increased significantly ($P<0.01$) by soybean meal in comparison with meat meal. The concentration of total phytoestrogen (TPE, $P<0.001$), daidzein (D, $P<0.005$) and genistein (G, $P<0.001$) were dramatically greater in the plasma of those hens fed soybean (as phytoestrogen source) compared with meat meal. Reduction in the enzyme activity and duodenal morphometry were found in the soybean compared with meat meal. Significant increases in calcium and protein retention with soybean meal at this stage corresponded with a greater expression of calbindin D_{28k} in jejunal tissue. In spite of these results, the role of phytoestrogen on capacity of the reproduction system and consequently on egg and eggshell quality,

needs to be clarified.

Secondly, 18-22 weeks old Leghorn pullets were fed similar diets to the last experiments (soybean and meat meal) and similar levels of estrogen (0 and 50 μg) to determine the effect of phytoestrogen on egg and eggshell quality. The result showed that no differences were detected in weight gain, daily feed intake and feed efficiency in regard to treatment. Also no significant differences were shown in the growth of the gastrointestinal tract with respect to treatment. The plasma concentration of TPE, D and G were significantly greater ($P<0.001$), ($P<0.005$) and ($P<0.001$) respectively, in those group* of hens fed soybean compared with meat meal. The concentration of TPE was higher than in the 14-week Leghorn pullets of the last experiment. Calcium ($P<0.005$) and protein ($P<0.001$) retention were significantly reduced by soybean meal in comparison with meat meal. It was concluded that phytoestrogen possibly blocks the activity of the reproduction system and consequently may reduce egg production in laying hens. Adverse effects were recognised on calcium retention, enzyme activity, villus characteristics, and caused a delay in egg production (14 days) as well as in eggshell quality (particularly eggshell percentage).

In conclusion, the existence of estrogen receptors in the jejunum tissue, particularly in the tip of villus which has the main role for calcium transport of pullets, indicates that there is a potential for direct effects of estrogen on the expression of calbindin D_{28k}, which is essential for calcium transport in laying hen. It was shown that the amount of estrogen normally present in the reproductive system is sufficient for the normal capacity of egg and eggshell production as no difference was observed in response between estrogen treated or control groups. The conflicting results in calcium and protein retention observed between 14 and 22 week-old-pullets when fed soybean meal may be due to the different intake of soybean. Consequently 22 week old pullets may

have consumed more phytoestrogen than pullets at 14 week of age. Thus more adverse effects occur in this particular situation. Egg production and shell quality were significantly reduced by phytoestrogen. The concentration of phytoestrogen in poultry diets needs to be investigated in more detail as it may act as an anti nutritive factor and effect* metabolism and productivity.

DECLARATION

I hereby state that this thesis does not contain material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief no materials previously published or written by another person, except where due reference is made in the text. I consent to this thesis, when deposited in the University Library, being available for photocopying and loan.

Ali Asghar Saki,

Date. 16/9/98

DEDICATION

This thesis is dedicated to my wife (Sahala) and to my children (Sahar, Mohammad, Milad and Sima) and my father whom I did not see again after I left IRAN to carry out this study.

ACKNOWLEDGMENT

I am indebted and most grateful of the following people for their assistance and support during the course of this thesis:

I wish to express my sincere thanks and deep sense of gratitude to my supervisor Dr. David Tivey for his constant encouragement, excellent advice, guidance, patience and constructive criticism over the period of experimental work and the preparation of the thesis. It was a great opportunity for me to work with him. I have learnt much from him over the period of study, (particularly he always asked me, why do you want to go and do that).

My sincere thanks to Prof. P. Hynd my co-supervisor and Head of Department for support and for providing facilities for my project. Many thanks to Dr. J. D. Brooker as coordinator of postgraduate studies and as a member of the review panel for his suggestions regarding my project. Many thanks also to other members of the review panel of my thesis Dr. Simon Maddocks for his excellent suggestions particularly on the summary of my thesis. I would like to thanks Dr C. Steeb for her support in immunohistochemical procedures and as one of the review panel members, and to Dr. W. S. Pitchford for his suggestion and guidance in statistical procedures used in my thesis.

I greatly appreciate and sincerely thank Dr. Brian D Siebert for his excellent advice and editing during the writing of my thesis and for his kind cooperation, and also to

Robert Kemp (PhD student) for his great assistance during the writing of my thesis and with computing.

My sincere thanks to Mr Reza Zarinkalam (laboratory technician) for his great assistance in experimental and laboratory work. Also many thanks to Natasha Penno as a helpful research officer. I would like to thanks of Mr. Rex Connolly (laboratory manager), and Mrs. Jenny Prosser, (departmental secretary) for their help, assistance and friendship and to all the staff and students (Paul, Peggy and Ross in the epithelial laboratory) in Animal Science.

I would like to express my appreciation to Mr Bob Hughes (President of World's Poultry Science in South Australia) Dr. van Barneveld and Dr P. Glatz for their assistance during the experimental work, chemical analysis of feed and in the supply of feed and animals. My thanks are also extended to Dr Fabian Dalais for his assistance regarding phytoestrogen measurements.

I wish to gratefully acknowledge the generosity of the Ministry of Culture and Higher Education of the Islamic Republic of IRAN for awarding a scholarship to allow me to pursue my studies at the University of Adelaide.

Finally I am sincerely grateful to my wife Sahala and my children Sahar, Mohammad, Milad and Sima for their faith, patience, understanding and for the great deal of the time they had to do without me.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

"Calcium requirement of laying hens is much greater than for any other species of domestic Animals"

Austic and Neesheim, (1990)

UNIVERSITY OF ADELAIDE
ROSEWORTHY CAMPUS LIBRARY

CANCELLED

1.1. Introduction

In 1992 the population of the world was 5.4 billion. It is now approximately 5.9 billion (U S Bureau, International Database, 1998) and it has been estimated that it will reach 10 billion by the year 2030. Such an explosion demands an increase in the world food supply (Harding, 1995).

Much of the world's human population suffers from malnutrition as well as from an unbalanced distribution of food. For example, in developing countries consumption of eggs and poultry meat can be as little as 20 and 240 g per head per year respectively. This compares with 260-300 eggs and 20-30 kg poultry meat per head in the developed countries (Banerjee, 1992). Thus eggs and poultry meat can be a major source of human food and research needs to be focused in an increasing poultry production. Accordingly, great effort is being made to study the possibilities of utilising new sources of feed as alternatives for domestic animals and not those which are utilised by humans (such as wheat). If new sources of feed can be found and processed by associated new technology, their ingredients need to be metabolised by cells in domestic animals. That means metabolism plays a basic role in feed conversion efficiency and as such determines the quantity and quality of poultry products. Consequently, the present study focuses on metabolic processes which control the utilisation of feed in laying hens.

One of the major nutrients which plays an essential role in laying hens is calcium, as it is critical in cell regulation and recently became a "hot" topic in the study of body functions, egg production and egg shell quality. It is in greater demand in laying hens than in other domestic animals (Austic and Nesheim, 1990).

Calcium deficiency leads to decreased growth and causes diseases such as rickets, as well as a decline in egg production by laying hens. Eggs are well known as an excellent source of energy, macro and micro nutrients (Noble *et al.* 1986). Therefore they play an important role in human nutrition (Christie and Noble, 1984). Eggs are not only a good source of food for humans but they play a vital role in developing embryos and chicken production in the poultry industry. In addition without adequate egg production, chicken production by the poultry industry would stagnate. The quality of the egg is as important to hatchability as it is to providing a source of food. It, in turn, is dependent on egg shell composition since poor egg shell quality will lead to high economic losses in the poultry industry.

Various estimates have been made of the financial loss caused by shell breakage. Cater (1971) and Hamilton (1982) as cited by (Hunton, 1987), have estimated a loss of £5 million in the U.K and $110 million for the U.S.A and Canada. In the estimates of egg production in the major producing countries as reported by the International Egg Commission, world-wide losses from egg shell breakage alone probably exceed US $ 600 million. This estimate does not include the USSR, South America and Asia (except Japan), (Sauvenur and Picard, 1987). An increase in the knowledge of calcium metabolism involved in egg shell quality should lead to a reduction in these financial losses, with these reductions, annual global loss due to poor egg shell quality has been estimated at US $ 500 million by recent investigations (Etches, 1996). However egg shell quality is still a major concern to the poultry industry in Australia where a loss of $ 60 million is incurred yearly (Sauvenur and Picard, 1987).

Eggs are protected from physical and pathogenic damage by egg shell quality which in turn is supported by minerals, particularly calcium. It is well established that the calcium requirement of laying hens increases dramatically during the sexual maturity and onset of lay (Bar *et al.*, 1990; Wu *et al.*, 1994). Indeed laying hens require two grams of calcium for each 60 g egg. This high demand for calcium can be influenced by vitamin D_3 ($1.25\ (OH)_2\ D_3$) during the process of Ca transport in the laying cycle. Transportation of calcium by the small intestine is modulated by changing hormonal status during sexual maturity (Deluca,1980; Hurwitz, 1989). It has been found that the production of vitamin D_3 ($1.25(OH)_2D_3$) which is a modulator of calcium absorption, is regulated by 1-hydroxylase enzyme in kidney. However, the production of this enzyme is affected by estrogen in the kidney (Elaroussi *et al.*, 1993 and 1994). The indirect effect of estrogen on calcium uptake is known, but there is a doubt of the direct action of estrogen in intestinal calcium transport. Nutritional and hormonal factors such as vitamin D_3, parathyroid hormone and estrogen as well as phytoestrogen in commercial laying hens diets may affect calcium transport in the intestine and therefore effect egg shell quality (Brody, 1994).

In conclusion, although most of the physiological roles of calcium transport are known, limited research has been conducted into transport mechanisms affected by the action of estrogen as well as phytoestrogens (as a major components of soybean meal) in pre laying hens (pullets). In addition, the interaction of different sources of feed (estrogenic and non-estrogenic feed) with calcium retention are also unknown. In particular, the permissible amount of phytoestrogen that will not adversely effect on egg production or eggshell quality needs to be determined urgently for poultry industry. The present study intends to address these issues.

1.2. Literature Review

1.2.1. Egg formation

During ovulation the yolk is released from the ovary and the mature ovum is collected by the infundibulum. The peivitelline* membrane of the yolk is formed in this part of oviduct. The chalazae, a white twisted structure which positions the yolk in the centre of the egg, is produced in the infundibulum region. Fertilisation takes place in this area. The yolk is maintained for half an hour in the infundibulum and then passes to magnum which is about 33 cm, the longest part of the oviduct (Roberts and Brackpool, 1994).

The major part the egg albumen (egg white), is made in the magnum. It has been found that albumen is a complex mixture of about forty different types of protein. Albumen processing takes approximately 2-3 hour in the magnum. Inner and outer shell membranes are rapidly developed in the isthmus which is narrower than magnum region. Eggs stay in the isthmus for nearly to* 2 hours and then travel into the tubular shell gland which is responsible for the transfer of calcium on to the membrane fibres (Hunton, 1995; Etches, 1996).

Calcium salts are deposited at the end portion of mammillary cores, which play a vital role of in shell formation. The egg next travels to the shell gland pouch. In this region calcium carbonate calcification takes place in the first four hours associated with water facilitated by some salts and glucose uptake. This stage is known as plumping and exaggerates the shell membranes (Roberts and Brackpool, 1994; Roberts and Brackpool, 1995).

At the beginning of calcification the shell gland fluid become more acid and then returns to normal when the shell is approximately half formed. After that, shell formation occurs and the egg resides for about 20 hours in the shell gland pouch (Petersen, 1965; Roberts and Brackpool, 1995). After the egg formation, egg quality is dependent on the quality of shell.

The eggshell has two basic and important roles, in protecting the contents, for example physical protection and preventing bacterial contamination. Therefore the normal egg shell should supply such characteristics. The shell as a container must be strong enough to support the processes of laying, collection, grading, transportation to market and handling by the consumer. It must also allow for the normal loss of moisture, oxygen and minerals and also sufficient physical protection of the embryo during the incubation period. The rate of hatchibility for chicken production during the hatching is dependent on shell quality and may influence on poultry production Etches, 1996).

The eggshell is a complex of organic and inorganic compounds composed of protein, carbohydrate, lipid, pigment and minerals, particularly calcium, in the form of calcium carbonate which is major component of the shell (90%) (Petersen, 1965; Sauvenur and Picard, 1987). Therefore for good shell formation, it is necessary to know the physiology of calcium in different parts of body, the rate calcium supplementation in the diet, calcium homeostasis, intestinal calcium absorption and transport, bone formation and mobilisation, kidney calcium reabsorption and endocrinal function and uterine calcium transport. The effects of the physical environment (rearing system, deep litter or cage and lighting), the genetic potential of

hens, diseases, drugs and water quality supply on calcium requirement and consequently on shell quality in the laying hens also can not be ignored.

The shell, obviously is the most visible and vulnerable component of the egg. Soft, thin, cracked and broken shells are caused by many factors, leading to losses during delivery, or total loss in cases where the egg fails to be collected.

A major reason for shell weakness is the amount of calcium deposited (Hurwitz, 1989; Roberts and Brackpool, 1995). This has been estimated by specific gravity, shell thickness, shell weight and shell weight per unit of surface. Deformation of the shell and shell strength also have been considered as other factors for measuring calcium and finally for shell quality. In addition to the above factors, shell strength most likely is dependent on construction of shell, because mammillary layers in the shell structure act as a foundation of shell formation (Roberts and Brackpool, 1995).

1.2.2. Structure and function of egg shell

1.2.2.1. The macrostructure and function of egg shell

The physical compartments of the various components of the eggshell and its content are shown in (Fig. 1.1.a, b). The composition of different part of eggs from various species are presented in the Table 1.1. Although some differences are observed in egg weight, albumen and yolk percentage, the eggshell constitutes 9-14 % by weight of the complete egg for all species.

The dimension and geometry of a standard chicken egg is illustrated in the (Fig. 1.2.a). The volume of egg (*V*) and the surface area of egg shell (S) can be calculated

from these dimensions and the egg's geometry (Etches, 1996; Narushin, 1997).

1.2.2.2. The microstructure and function of egg shell

The shell is composed of several layers that function together as a single unit to serve as the embryonic lung during development, as a container of the contents and also a barrier to prevent the entry of micro-organisms into the egg (Figure 1.2.b). The shell consists of outer and inner membranes, mammillary layer, palisade layer, surface crystal layer and cuticle (Fig. 1.1.b and 1.2.b).

The inner shell membrane is in contact with albumen in the egg and is made of parallel fibres layers. These fibres of restrict the passage of micro-organisms into the egg. The outer shell membrane is about 3 times thicker and tighter than the inner shell membrane (Austic and Nesheim, 1990; Etches, 1996). The membranes normally adhere to each other except at the large end of egg, where they are separated to form an air cell. Both membranes are entirely permeable to water and air. The fibres of the outer shell membrane are attached to the shell in areas called mammillary cores (Roberts and Brackpool, 1995).

Table 1.1: The composition of eggs from various species of poultry

		Egg composition (%)		
Species	**Egg weight (g)**	**Albumen**	**Yolk**	**Shell**
Chicken	58	56	32	12
Turkey	85	53	33	14
Guinea fowl	40	52	35	13
Pheasant	32	53	35	12
Japanese quail	9	58	33	9
Goose	155	56	32	12
Pekin duck	92	57	33	10
Muscovy duck	80	53	35	12

From Romannoff and Romanoff (1949) and Sauveur (1988) cited by Etches (1996).

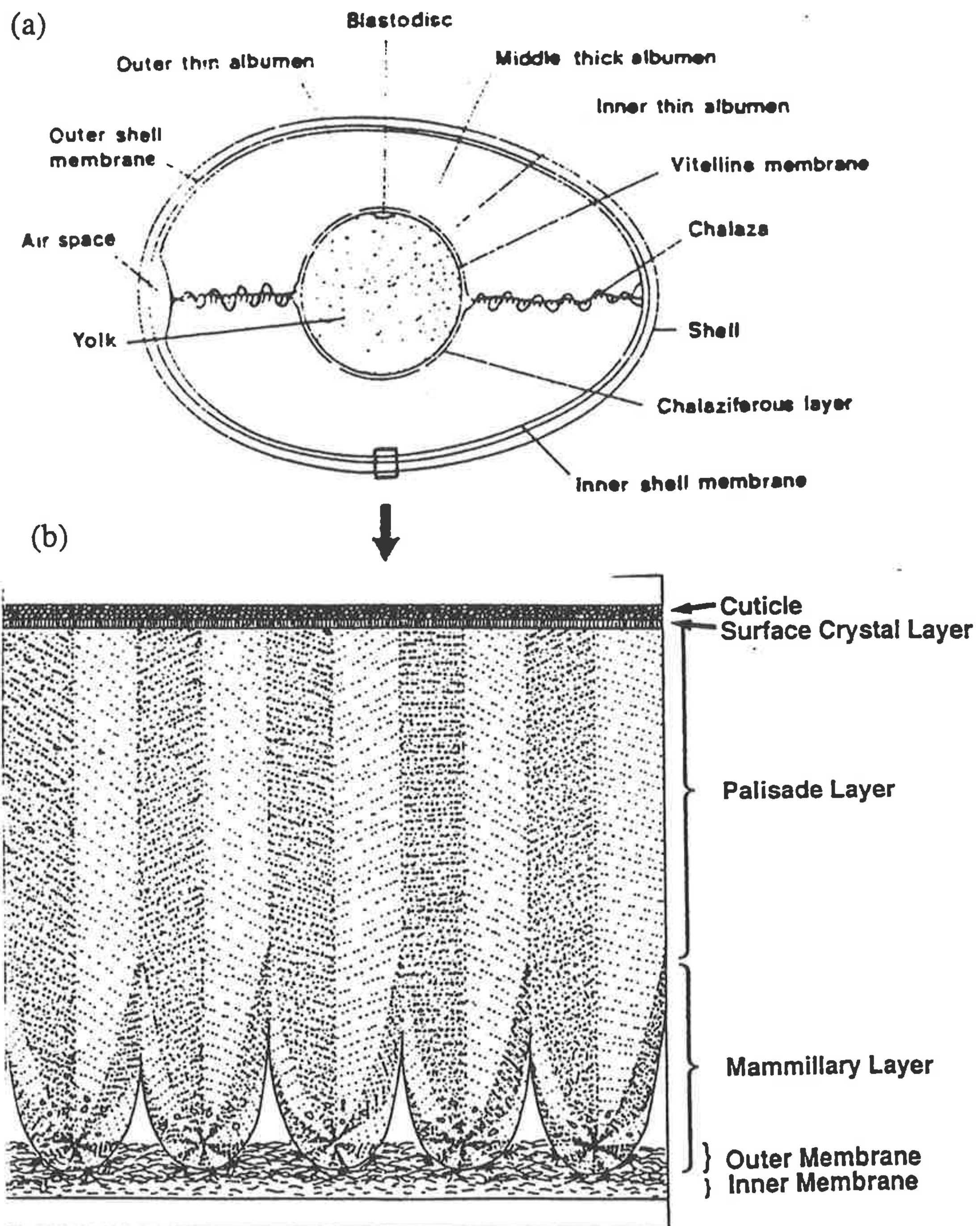
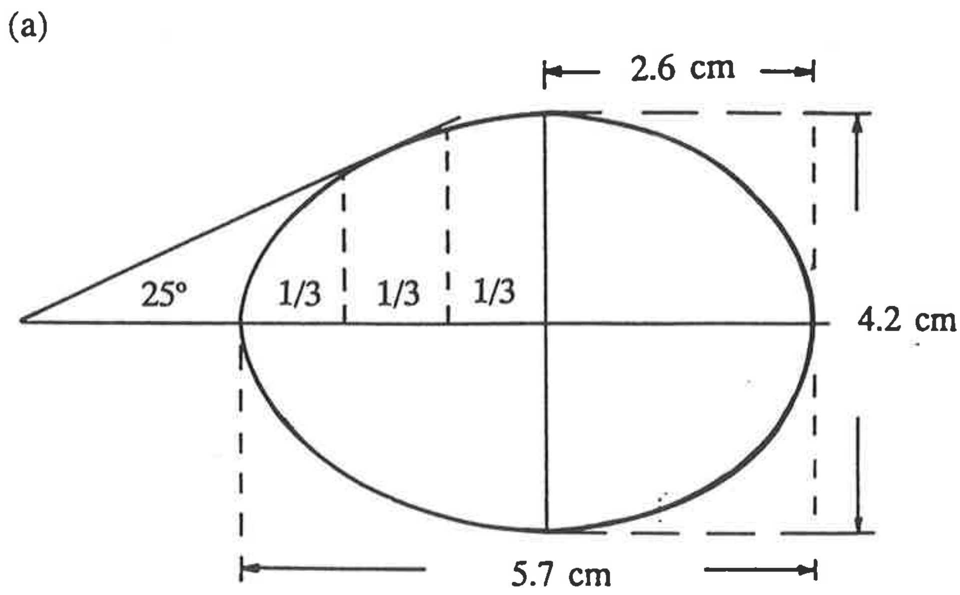


Figure 1.1 a, b: The physical components of egg and egg shell; (a, from, Tullet, 1987; b, from, Robert and Brackpool, 1995).

Small granules are deposited in the outer shell membrane in the surface of shell membrane. These are formed in the center of mammillary cores and become the initial calcite crystal layer. In the first stage of calcification, liquid penetrates between the membrane fibres of the outer shell membranes. Therefore the inner part of mammillary layer becomes embedded in the outer shell membrane (Roberts and Brackpool, 1995).

The spongy or palisade layer starts its formation on the mammillary layer. It seems to be that there are two layers are formed in the palisade layer during the development and growth deposition stage, the inner layers is more dense while the outer is more porous. The growth of the palisade layer is achieved by the addition of one layer over another and may be terminated by an organic matrix in the surface crystal layer. Although the mammillary layer can contribute very little to the stiffness of the shell, the lower part of the palisade layer is responsible for 50% of the strength of the complete shell (Etches, 1996). Most of the $CaCO_3$ that in deposited is formed in this region (palisade layer). The thickness of both layers (mammillary and palisade) is approximately 300 μm (Figure.1.1 b and 1.2 b), (Roberts and Brackpool, 1995; Hunton, 1995; Etches, 1996).

The surface of crystal layer is located on the palisade layer and varies in thickness. Calcium is transferred from the shell glands to the crystal layer and this may be the reason for a transition of calcium from the palisade crystal columns to polycrystalian in surface of crystal layer (Figure 1.1 b), (Roberts and Brackpool, 1995; Etches, 1996).



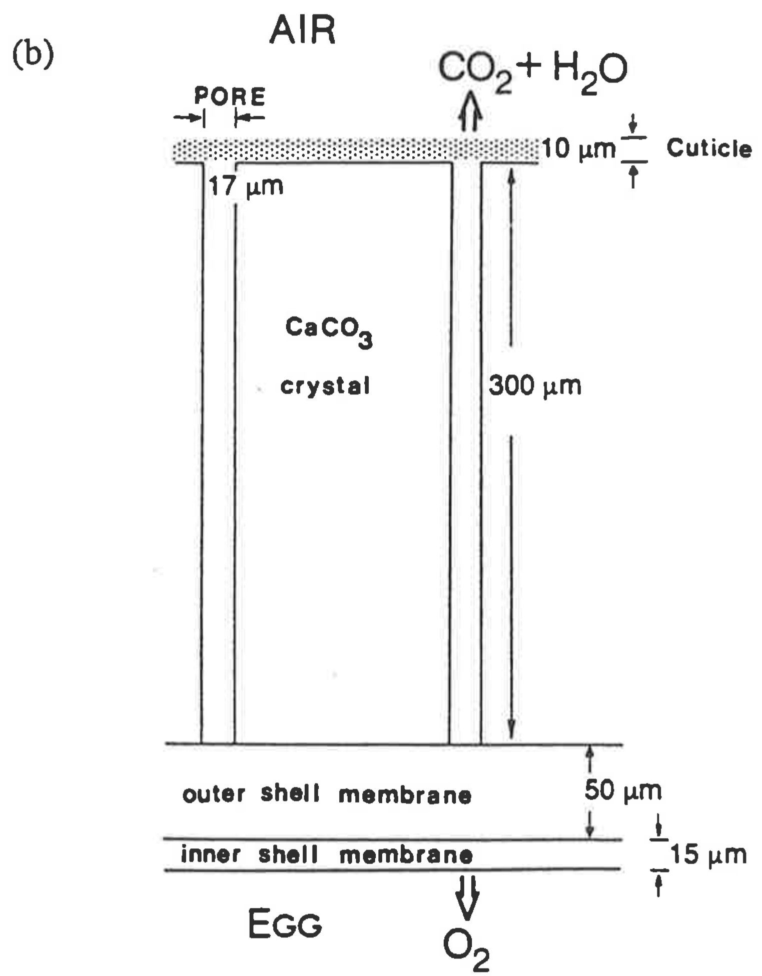


Figure 1.2(a, b): The dimensions and geometry of hen's egg, From Romanoff and Romanoff (1949) cited by etch, 1996; (b) Representation of the anatomical structure of the eggshell and the flow gases through the pores, from Etches, 1996.

The cuticle is a thin waxy layer containing protein, polysaccharide and lipid which is secreted while the egg is being laid. This layer adds little to the strength of shell and helps to prevent entry of bacteria into the egg and also slows the rate of water loss from the egg. The formation of shell pigment had been shown to occur in this layer but still there is doubt to whether this pigment comes from the blood or shell gland fluid and also there is still doubt related the effect of pigment on shell strength and thickness (Roberts and Brackpool, 1995; Hunton, 1995; Etches, 1996).

1.2.3. Estrogen, egg and egg shell quality

The physiological role of estrogen (E_2) as a female sex hormone in sexual distinction, development of the reproductive tract and regulation of the reproductive system in adults has now been defined (Korach *et al.* 1996; Klandorf *et al.* 1998). It has long been recognised that estrogen increases during sexual maturity in laying hens (Hurwitz, 1989). The development of secondary sexual characteristics is also accomplished by endogenous estrogen in laying hens (Lien *et al.*, 1985). The increase fat to supply lipid for yolk and calcium for shell formation were coupled with estrogen secretion during the sexual maturity (Turner, 1948; Adams *et al.* 1950).

During the onset of lay in hens an increase in Ca absorption is associated with an increase in plasma vitamin D_3 ($1.25(OH)_2D_3$), (Hurwitz *et al.*, 1973; Bar *et al.*, 1978, cited by Qin and Klandrof, 1993). Production of vitamin D_3 is strongly dependent on the action of estrogen to produce the enzyme 1-hydroxylase in the kidney (Bar and Herwitz, 1972; Bar *et al.*, 1978, cited by Qin and Klandrof, 1993). In addition to the indirect effect of estrogen on calcium metabolism, * is now thought that there is a potential direct action of estrogen on intestinal calcium transport (Arjmandi *et al.*,

1993). Consequently the ability of estrogen to modulate calcium absorption from the small intestine of laying hens, may effect* egg production and shell quality (Qin and Klandrof, 1993)

Both endocrine and transport functions are basic roles of kidney in mineral homeostasis during the laying cycle of hens (Elaroussi *et al.* 1993). The activity of 25(OH) D_3-1-hydroxylase in the kidney increases in laying hens and is responsible for increases in circulating of 1,25$(OH)_2D_3$ (Castillo *et al.*, 1979; Martz *et al.*, 1985). Administration of E_2 or E_2 in combination with the other hormones such as testosterone or progesterone effects* on calcium transport similarly, although intestinal calcium absorption is not effected* by the direct effect of estrogen in birds (Baski and Kenny, 1981). Previous studies have shown that E_2 can increase the synthesis of 1,25$(OH)_2D_3$ in the kidney by inducing the activity of the 25,(OH)-1-hydroxylase enzyme. This may be the reason for increased expression of intestinal calcium binding protein (calbindin D_{28k}), (Bar and Hurwitz, 1979; Sommerville *et al.*, 1989). In contrast, other hormones such as follicle stimulating hormone (FSH), cortisone, testosterone and progesterone even at high levels, produce little or no change in 25,(OH)-1-hydroxylase enzyme (Castillo *et al.*, 1976). As mentioned before E_2 may induce calbindin D_{28k} in the intestine.

In addition CaBP (calbindin D_{28k}), has been shown to play a vital role in Ca transport from the serosal side of epithelium of the small intestinal (Pansu *et al.*, 1989). Recent studies have suggested that laying hens with a reduced intake of vitamin D_3 or Ca, have a reduced rate of estrogen secretion (Tsang *et al.*, 1988). This point again demonstrates the complex relationship between estrogen, Ca and vitamin D_3 metabolism (Sommerville *et al.*, 1989). Most of the fundamental information

concerning estrogen function has shown that estrogen is a modulator of vitamin D metabolism with the other known regulator, parathyroid hormone (PTH) (Pike *et al.*, 1978). Arimandi *et al.* (1993) have reported that estrogen has a physiological role in the regulation of intestinal calcium transport in postmenopausal women This might also occur in the laying hens.

The uptake and response to estrogenic compounds administrated by injection, capsule implantation or oral dose by either capsule or by mixing in feed was examined by Lien *et al.* (1985). It has been found that injected estrogen was approximately 100 times more potent than dietary estrogen. In contrast, administration of estrogen by short term injection could not produce a steady concentration of the hormone (Qin and Klandrof, 1995). Long term injection may over come this problem. Also because implants are bundled together, they have limited contact with the surrounding tissue which may prevent the absorption of estrogen into the body of hens. In addition the release of estrogen from an implant capsule does not alter with changes in body weight gain during the experiment (Qin and Klandrof, 1995), resulting in variability in estrogen dose on a body weight basis.

There are conflicting results regarding the effect of exogenous estrogen on egg production and egg shell quality in aged White Leghorn hens. while certain studies (Gunder *et al.* 1980; Gunder *et al.* 1981) have shown an increase in egg shell thickness, egg weight and oviduct weight in 32 week old hens receiving 17-β estradiol, no response has been observed in others studies (Li *et al.*, 1986; Qin and Klandrof, 1995). However the effect of age should not be ignored in this matter. Increase in total plasma calcium concentration is associated with an increase in the protein bound fraction in response to increase vitellogenin synthesis in the liver

which is induced by estrogen (Gunder *et al.*, 1978 Guyer *et al.*, 1980). There are differences in the synthesis or degradation of vitellogenin due to genetic potential of laying hens. This could be related to estrogen effects in thick and thin shell birds (Gunder *et al.* 1980). Although Hurwitz, (1989) concluded that vitellogenin is the yolk precursor substrate, an increase in plasma calcium before egg laying (during the yolk formation) is not related to egg shell formation. Therefore, the affect of vitellogenin on calcium metabolism in laying hens and consequently on egg shell quality needs to be clarified.

1.2.4. Structure and function of estrogen receptors

The estrogen receptors (ER) is a nuclear protein and one of a member of the steroid receptor superfamily. These nuclear hormone receptors are complex as a ligand-activated transcription factors and bind to DNA which produce responses that control cellular processes (Evans, 1988; Beato, 1989; Schwabe *et al.*, 1993; Parker, 1995; Korach *et al.*, 1996). Comparison between amino acids sequences of human and chicken shows that 80% of them are identical (Krust *et al.* 1986), and estrogen receptors have been identified in the nuclear and cytoplasm of uterus cells in laying hens using antibodies raised against human receptors (Sutherland and Baulieu, 1976; Kon *et al.*, 1980; Yoshimura *et al.*, 1995).

All classes of steroid receptors are recognised to be nucleocytoplasmic transporting, in fact they appear to diffuse into the cytoplasm and rapidly return to the cell nucleus (Parker, 1995). Recently investigations have been made to determine whether that tmoxifen and the other antiestrogen such as ICI 182780 may compete with the estrogen receptor and bound to DNA. This could blocked the transcriptional activity

which is mediated by estrogen receptor (Parker, 1995). Dichloro-diphenyl-trichloroethane (DDT) and polychloronated biphenyl (PCB) are also associated with production of thin egg shell and are known to be environmental pollutants (Chen *et al.*, 1994). They may compete with estrogen receptor to binding in DNA and as a result produce thin shells in laying hens (Chen *et al.*, 1993)

Estrogen receptors present in tissues (e.g. various cells and tissues of the uterus) are affected by the specific action of estrogen. DNA and RNA, protein synthesis, mitosis and prolactin synthesis are increased by estrogen whereas, FSH and LH production are suppressed by this hormone (Simmen *et al.*, 1984; Quarmby and Korach, 1984 a,b and Rosenfeld *et al.*, 1987, cited by Korach *et al.* 1996;). In addition to the activity in uterine tissues, recent studies have indicated that estrogen acts on other target sites in bone (Ettinger *et al.*, 1985, cited by Korach *et al.*, 1996) and the small intestine (Arjmandi *et al.*, 1993). It is difficult to fully understand the action of estrogen in these tissues (bone and intestine) due to the lack of a suitable physiological model system

1.2.5. Calcium function, calcium dietary, requirement and homeostasis in pre-laying hens.

1.2.5.1. Calcium function in pre- laying hens

Calcium is quantitatively an important inorganic element in the body, being one of the principal mineral constituents of the skeleton which contains 99% of the total body calcium (Sallis, 1962).

Many cell and organelle functions maintain extra cellular fluid calcium concentration within a very small range. Several important metabolic actions are affected by slight

changes in the concentration of intra and extra cellular ionised calcium. These include: (1) the sensitivity of nerve function and activity of cell membrane transformation, (2) the secretion of proteins, hormones and other substances by the cell, (3) the affinity of cell excitation and responses such as the contraction of muscle cells and secretions in secretory cells, (4) the formation or development of cells during the growth, (5) coagulation of blood, (6) cell membrane stability and permeability, (7) enzyme regulation and (8) bone mineralisation (Mundy, 1990). All of these functions are very important in the poultry body, particularly at the layer stage, because the concentration of calcium in the isthmus falls from about 77 mg Ca/g ash to about 38 mg Ca/g ash as the egg passes through this region (Simkiss and Tayler, 1971). Also symptoms of calcium deficiency in laying hens are coupled with (a) reduction of growth, (b) reduced feed consumption, (c) increased basal metabolic rate, (d) decreased activity of laying hens (e) osteoporosis and rickets (f) increased urine volume (g) decreased life span, (h) reduced egg production and shell thickness, (i) demineralisation of bone, (j) reduced serum calcium concentration and (k) decreased calcium and magnesium in the carcass (Scott *et al.*, 1982)

The demand for calcium is increased during the egg production and this must be achieved by a dramatic increase in absorption from the diet by the gut. Calcium not only serves as the principal component of the skeleton, but participated in the structure entirely essential to support the increasing body size of the individual during growth as well as being associated with essential physiological and biochemical processes. Thus, maintenance of circulating calcium within a narrow range is critical for animal's survival (Hurwitz, 1989).

1.2.5.2 Dietary calcium supplementation

Absorption of dietary calcium by the gut is the major way that increased body calcium stores and can be accumulated and maintained. Egg production and also eggshell quality are related predominantly to calcium from the diets. Supplementary calcium is supplied in diets in the form of particulate or pulverised limestone, marble or shell chips (Hughes, 1972; Taylor *et al.*, 1995). Particles of calcium must be sufficiently large to allow fragments to remain in the gizzard throughout the night (a time during which laying hens do not eat, as they need to be in the dark for photoperiod regimes). The particles must be sufficiently soft and large enough surface to allow the gastric acidity to dissolve them at rate that will release calcium for absorption into the blood stream (Miller and Sunde, 1975; Ronald *et al.*, 1978; March and Amin, 1981). In addition, the most active period of shell formation happens during darkness. The regime should remain at the standard, 14 hours light 10 hours dark in photo schedules for regulation of egg production (Etches, 1996). Feed consumption increases two hours before the onset of darkness, and appears to be stored in the crop at onset of darkness. If at this time diets can contain sufficient calcium, hens will consume large amount of the mineral during the two hours before the dark period and temporarily store Ca in the crop. Large amounts of calcium sufficient, to meet the demand of shell formation, are regulated throughout the period of darkness, provided the calcium passes from the crop to the lower part of digestive tract (Scott *et al.*, 1982; Etches, 1996).

Calcium absorption from the intestine of laying hens to the extracellular fluid is not only related to calcium and vitamin D_3 in the diet but is also associated with other dietary nutrients such as lactose, glucose some cationic amino acids (such as lysine) and fatty acids which increase calcium transport. On the other hand phosphate,

xylate and phytate decreases calcium uptake (Patton and Sutton, 1952; Vaughan and Filer, 1960; Civitelli *et al.*, 1992). Blood calcium concentration increases by 40% during the absorption of calcium from the intestine, while its concentration falls in the oviduct of laying hens during egg formation (Simkiss and Tayler, 1971). Deficiencies of calcium and vitamin D_3 in the diets can lead to decreased plasma calcium concentration in the laying hens and this could effect* on shell quality (Ruschkowski and Hart, 1992).

Increasing the Ca level from 3.0 to 4.5% and from 7.5 to 9% in diets produces significantly greater shell strength (Jackson *et al.*, 1987). In contrast, dietary Ca restriction reduced shell weight, shell density and structural bone and plasma calcium in both young and aged birds (Bar and Hurwitz, 1987). However, laying hens consume approximately 25% more feed during eggshell formation than non-laying hens and this is associated with increase* calcium intake associated the egg production. In addition, different concentrations of calcium in poultry diets may affect the delivery of calcium to the body of laying hens. When calcium concentration in diets is 3.56% or higher, most eggshell calcium is derived directly from the intestine (Simkiss and Tayler, 1971), and this may be regulated by estrogen. If the dietary calcium concentration is 1.95% or lower, bone supplies 30-40% of the shell calcium. The role of PTH and vitamin D_3 for the release of calcium from the bone in this pattern is important (Simkiss and Tayler, 1971; Nemere *et al.*,. 1984).

1.2.5.3 Calcium Requirement

Calcium requirement of the pre-laying hens can be supplied by different ways (Fig. 1.3). Approximately 2 grams of calcium is used for every eggshell formed by laying hens. Hens producing approximately 250 eggs per year need to receive 500 grams of

calcium to deposit this amount in their eggshells each year. This calcium is deposited as calcium carbonate in the eggshell. However, only 50-60% of calcium consumed can be retained in the eggs (Austic and Nesheim, 1990), thus within the shell formation 1000 g of calcium is required per year. This illustrates, that the calcium requirement of laying hens is much greater than for any other species of animal (Austic and Nesheim, 1990).

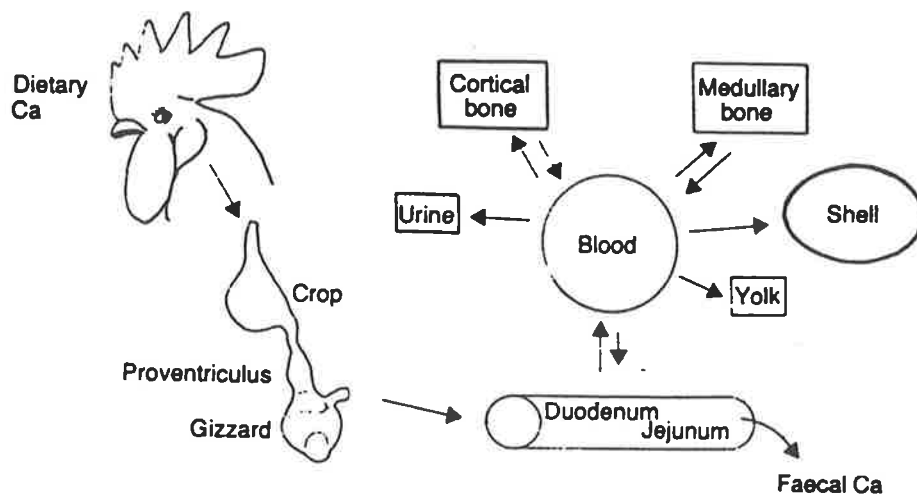


Figure 1.3: Process of calcium requirement in the laying hen. Modified from Etches (1987), cited by Etches 1996.

Calcium requirement is different between young and old laying hens. It is quite possible for young pullets in their early stage of production to form a good shell with less than 3% calcium in the diet, although in the early stage of egg production birds are in a calcium negative balance and this may continue for the first half of the laying

year (Petersen, 1965). It is noted by Hurwitz (1989) that at the onset of lay, energy supplied by feed intake is too slow (energy utilisation and metabolism rate of energy) to provide for the increased energy demands. As a result, a temporary calcium deficiency, regarding to the lack of energy occurs. Although during this time calcium storage in bone may assist shell formation, this situation can not be maintained for more than two weeks (Hurwitz, 1989). Calcium requirements in immature birds increase greatly during sexual maturation as birds lay down calcium in the bone and later start to produce eggs (Simikiss, 1961). In addition, feed calcium percentage of old laying hens may be increased to as much as 4 to 4.5% particularly in hot weather to improve eggshell quality. However, Petersen (1965) reported that high calcium intake in aged hens will not prevent a reduction in shell quality, this may be in part due to a limited ability of laying hens to retention dietary calcium.

Calcium requirement in laying hens is much greater than in non-layers and is dependent on age and egg calcification. As stated above, hens receiving 3.5-4.0% calcium in all mash rations have been shown to retain only about 50% of the ingested calcium (Giminigar, 1961; Mongin and Sauveur, 1974). A hen taking in 3.6 g of calcium per day retains about 1.8 g of calcium during the 18 hours that feed is available. Thus a hen retains about 100 mg of calcium per hour. This is supported by studies showing that calcium absorption is in excess of 100 mg per hour when the egg is in the uterus, but much lower when no eggshell is being deposited (Hurwitz and Bar, 1965; Hurwitz and Bar, 1969). This amount of calcium must be provided in the majority by diets. If large particles of calcium carbonate in the form of oyster shell are substituted for a portion of the pulverised calcium carbonate in the diet, eggshell quality may be improved by supplying the hen with dietary calcium 24

hours per day. Absorbing calcium at the rate of 100 mg per hour for 24 hours, the hen should then be able to retain 2.4 g of calcium, as slightly more than the 2.0-2.2 g are needed to make a good eggshell.

It has been found that there is a direct relationship between the calcium requirement and age in laying hens (Hurwitz and Bar, 1971). * National Research Council (1994) reported that the calcium requirement in immature laying hens is lower (1-1.2%) than in aged hens (3.3-4%), although a difference in feed intake can change the percentage of calcium requirement in the laying hens (National Research Councel, 1994). The calcium requirement of laying hens can be determined by application of the knowledge of the calcium content of the egg, the maintenance requirement for calcium, body size, the amount of feed consumed and the energy content of the diet, the rate of egg production and environmental temperature. The following is an example of calcium requirement of the laying hens.

Calcium content of large eggs with good shell quality	2.2 g
Calcium need for maintenance	0.1 g
Total calcium needed/egg/day	2.3 g
Average retention of Calcium	50 %
Dietary calcium/ hen at 100% production	4.6 g

$$Ca / hen / day = \frac{(EggCa, g \ x \ \% \ rate \ of \ egg \ production)}{(\% \ Ca \ retention)}$$

$$\% Ca \ required = \frac{(\% \ Rate \ of \ production)}{(lbl. feed \ consumed / 100 \ hens)}$$

$$\% \ Ca \ retention = \frac{(Ca \ retention, \ g \ x \ 100)}{(Ca \ intake, \ g)}$$ (Petersen, 1965).

1.2.5.4 Calcium homeostasis in pre-laying hens

Homeostasis of calcium in the blood and extracellular fluid of hens represents one of the most interesting biological control systems in poultry and is achieved by the interaction of a variety of hormones activity through the skeleton, kidney and intestine. It is evident that the availability of dietary calcium is an important determinant of calcium homeostasis (Civitelli *et al.*, 1992).

Electrolyte balance between cations and anions in the blood is absolutely necessary for homeostasis in all animals and poultry. The major cations in the blood plasma are sodium and calcium; potassium and magnesium are present also. The main anions are chloride, bicarbonate and phosphate. The involvement of acid-base balance in shell deposition arises from the production of hydrogen ions in carbonate formation (Muller *et al.*, 1969). Bicarbonate and pH are also significance in the formation of the egg shell (Hughes, 1989; Muller *et al.*, 1969). Amino acid and protein, being amphoteric, may serve either as cations or anions, depending upon the ratio of carboxyl to amino groups in the amino acid or protein (Scott and Zeigler, 1963). These essential electrolytes (calcium, potassium, magnesium etc) together with amino acids and protein maintain proper blood acid-base balance for the normal functioning of all body cells. They also play an important role in shell formation (Mongin, 1968).

Any factors which may change the relationship between cations and anions in the blood, therefore, may have a marked effect upon its bicarbonate level, thereby either reducing or increasing the amount of carbonate ion available to the shell gland for calcification of the eggshell. These factors include high temperature (Hamilton and Thompson, 1980; Harms, 1982), atmospheric carbon dioxide (Frank and Burger,

1965) and ascorbic acid (Njoku and Nwazota, 1989). These factors modify the amount of the major component laid down in the eggshell, calcium carbonate (Mongin, 1968; Eastin and Spaziani, 1978).

Calcium continuously enters the extracellular fluid, from the gastrointestinal tract and bone, but calcium concentration in the extracellular fluid is maintained relatively constant by the effect of inter dependent hormonal mechanisms, that regulate the influx and efflux of calcium between extracellular fluid, bone, kidney and the gut (Deluca, 1980; Panus *et al.*, 1983; Mundy, 1990).

The important regulator of calcium homeostasis of extracellular fluid calcium concentration within 7.5 and 11.5 mg/dL is almost certainly parathyroid hormone (PTH), but 1,25-dihydroxyvitamin D_3 is also necessary to maintain normal plasma calcium (Deluca, 1980; Mundy, 1990; Brody, 1994). Secretion of PTH by the parathyroid gland is stimulated by a fall in plasma calcium. The hormone stimulates bone reabsorption and activates the 1-hydroxylase enzyme which can convert 25-hydroxyvitamin D3 to 1, 25 dihydroxvitamin D_3 (1,25$(OH)_2D_3$) in kidney (Fig. 1.4). Therefore the increased 1,25$(OH)_2$ D_3 in the kidney provides increased calcium binding protein (CaBP) by interaction of 1,25$(OH)_2D_3$ with vitamin D3 receptors in the intestine (Berdanier and Hargrove, 1993). The vitamin D system in calcium homeostasis is discussed by (Deluca, 1979). Intestinal and uterine calcium binding protein (calbindin D_{28k}) synthesis are modulated by 1,25$(OH)_2D_3$. Consequently, calcium absorption is regulated by these processes in the intestine and uterus (Wasserman, 1981).

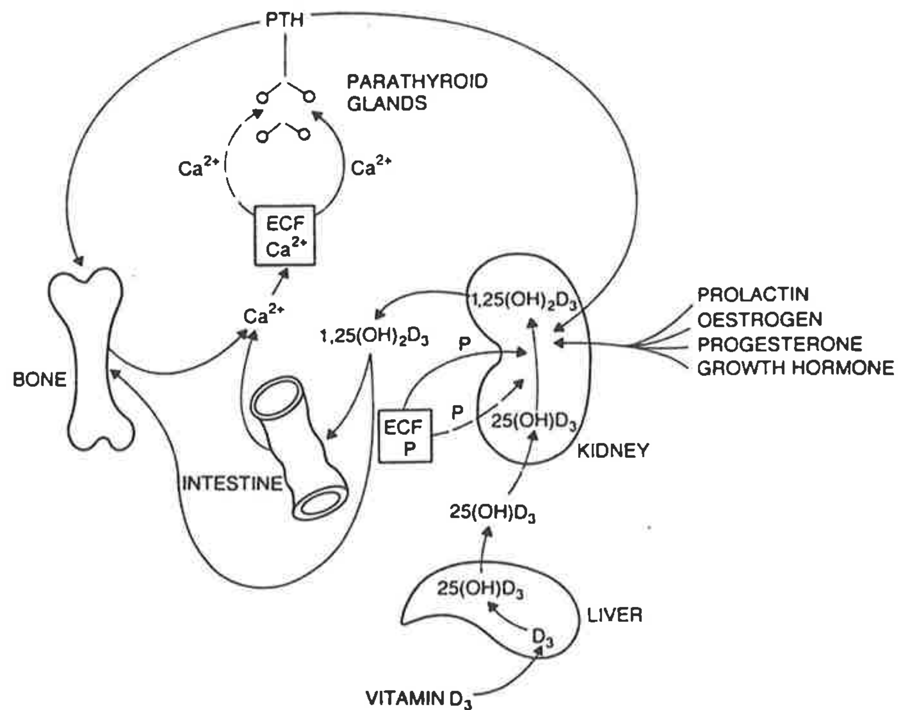


Figure 1.4: Illustrated calcium homeostasis in laying hens, from, Drezner and Harrelson (1979), cited by Etches 1996.

It is suggested that renal transport system in birds is more important than in other species, since only 0.5-2% of calcium is handled by urinary excretion in the rat and human kidney but close to 10% of an * absorbed calcium is lost in urine of birds (Hurwitz, 1989). This result shows a large excretion of ingested calcium in laying hens * can be reduced calcium during the shell formation (Hurwitz and Griminger, 1961).

The capacity of the kidney to excrete and the capacity of exchange between extra-and intra-cellular fluids can determine the stability of plasma calcium. Plasma calcium is maintained in the normal range by optimum outflow capacity of the kidney in

combination with the bone exchange mechanism. Therefore, calcium homeostasis is related to the concentration of calcium in the extracellular fluid which is determined by the capacity of the outflow system (both kidney and bone) to match the change with inflow (intestinal calcium uptake). Therefore, absorption of calcium from the small intestine and also calcium reabsorption from the kidney and bone are the major components involved in calcium homeostasis (Deluca, 1980; Panus *et al.*, 1983; Mundy, 1990; Smith and Deluca, 1993).

The reproductive system in laying hens during the shell formation has a high requirement for calcium (Gilbert, 1983). Most of the calcium comes from the diet and after feeding the intestine plays an important role. The quantity of calcium ingested depends on the amount in the diet and the appetite of the laying hens, the latter being affected by the stage in diurnal laying cycle, egg and egg shell formation. Calcium transport in the intestine is regulated by the stage of reproduction especially estrogen hormone concentration, age, bone reabsorption and diet of the hens as well as by diurnal factors (Mannion and Reichmann, 1984). The maintenance of the plasma ionic calcium concentration, takes priority over other processes such as bone and eggshell calcification (Sauvenur and Picard, 1987).

1.2.6. Regulation of intestinal nutrient transport

The intestine as a major part of the gastrointestinal tract with particular compartments play specific functions in nutrients digestion and absorption. Nutrients, electrolytes and water supplied by the intestine support the body structure and functions of animal.

Although absorption of nutrients by the intestine (sugar, amino acid, peptides, water-soluble vitamins and minerals) is modulated by diet or body stores of these nutrients, two types of mechanisms are known in absorption. One is a specific type, the numbers of transport site can be changed in the enterocyte. Therefore, the rate of protein synthesis or degradation will effect the transport capacity. The changed number of Na-glucose cotransporters in the brush border membrane is a good example for this aspect (Ferraris, 1994). A second, the non-specific absorption of nutrients are mechanisms that are not specific to transport any given nutrient in the small intestine (Ferraris, 1994). Calcium transport as a specific nutrient in the intestine of laying hens may have particular mechanisms for release from the diet and absorption during the laying cycle.

1.2.6.1. Intestinal structure and function

The mucosa absorptive surface of the small intestine consists of a large number of villi with in a cm^2 of duodenum containing about 10^8 cells (Harme and Wright, 1980). Each villus contains a layer of epithelial cells enhanced by a brush border of microvilli. These microvilli extend into the lumen of small intestine to increase the surface area (Alberts *et al.*, 1994).

The potential of the gastrointestinal tract (GIT) to digest and absorb nutrients has been assessed by measurement of the total epithelial surface exposed to nutrients. This is related to the length and surface area of the villus in different regions of the small intestine. In addition, the ability of the small intestine to express enzymes activity and transport proteins in brush border membranes should be noted in this matter. Stem cells in the crypt area produce epithelial cells for the villus (mitotic area, Fig.1.5) (Smith and Peacock, 1989; Alberts *et al.*, 1994). The differentiated

cells are created from these stem cells and move to the exposed regions of the villi. At the end of this process, the cells die at the tip of villus and then sloth off into the intestinal lumen (Alberts *et al.*, 1994). The cells develop their brush border membrane during the migration from the crypt area to the exposed areas where digestive enzymes are formed. A transport stage follows after these steps and nutrients transported by the brush border membranes (Smith, 1990; Ferraris, 1994). However, finally the absorbed nutrients can be processed by intestine epithelial cells.

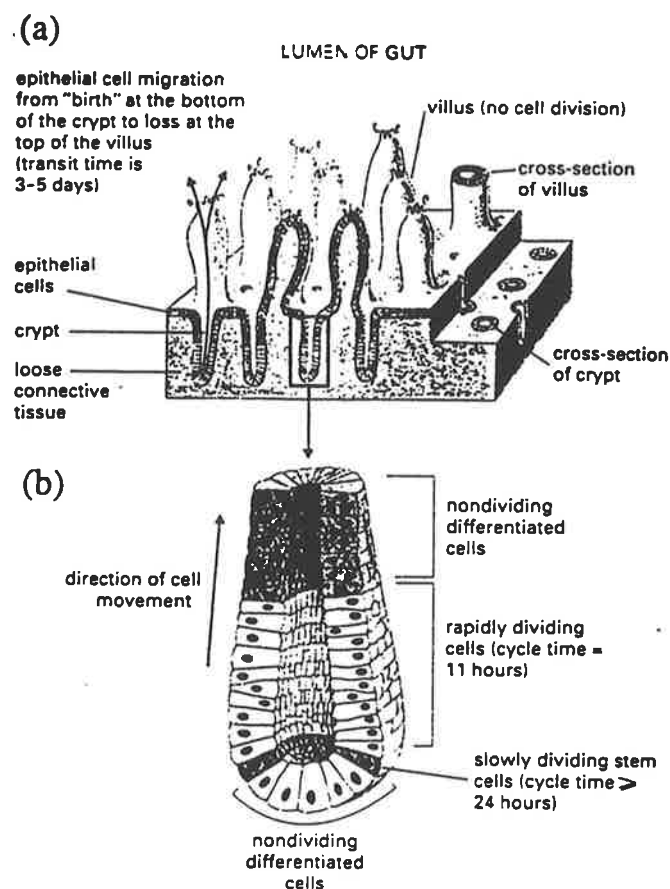


Figure 1.5 (a, b): **Cell division and migration in the epithelium of small intestine mucusa.** from Schofield and Lojtha, Biochima and Biophsica Acta, 1979, cited by Robert (1994).

The intestine epithelial cells have the potential to absorb non-electrolytes (sugar, amino acids, water) and electrolytes (sodium, potassium, chloride, bicarbonate). This capacity is achieved from the particular structure. In the architecture of epithelial cells, they attach to each other by a structure called tight junctions. The cell organisation is given additional structural richness by a glycoprotein matrix called the basement membrane. The membranes with two distinct parts, produce a polarised cell (cell with two opposite site) and tight junctions combine to allow the epithelium to transport both electrolytes and non-electrolytes. There are two transport pathways, first across the tight junction and along lateral intercellular spaces; the paracellular pathway. Secondly across of the apical membrane into the cell, followed by the exit across the basolateral membrane and this is referred to as the transcellular pathway (Nancy *et al.*, 1996).

Epithelial cells contain three basic building components: (1) Tight junctions that bind the cells together and may restrict the movement of substance between the epithelial cells, (2). The apical membrane separated from the basolateral membrane by tight junctions, (3) The gap junctions provide communication from cell-to-cell. The spaces between the cells is the lateral intracellular space (Fig. 1.6) (Nancy *et al.*, 1996). Epithelial cells overlay the villus structures of small intestine which can change dramatically in response to different types of feed

Recently, attention has focused on the effect of various diets on intestinal morphology (Brunsgaard and Eggum, 1995; Yu and Chiou, 1996). It has been found that polysaccharides are one of the best stimulators for rat gut proliferation, particularly non-starch-polysaccharides (NSP) which have a very strong effect on intestinal structure (Brunsgaad *et al.*, 1995). Microvilli are a complex structure

consisting of about 20 actin filaments bound to each other and to a number of proteins within the plasma membrane, some of which change their characteristics when exposed to NSP and calcium (Smith *et al.*, 1984; Van coven *et al.*, 1985). These data lead to the conclusion that intestinal morphometry can be influenced by diet.

1.2.6.2. Calcium transport in the small intestine

Calcium transport is most efficient in the first part of the small intestine in particular duodenum (Civitelli *et al.*, 1992), but the majority of calcium is mainly absorbed in the * in the upper part of jejunum (Sallis, 1962; Gilbert, 1983; Hurwitz, 1989). The absorption of calcium represents the summation of two processes: (i) the total calcium absorption from intestinal lumen to plasma and (ii) the total transfer of calcium from plasma to lumen, or endogenously secreted calcium (Mundy, 1990; Civitelli *et al.*, 1992).

Calcium absorption by the intestine is mediated by both paracellular and transcellular pathways (Bronner, 1991; Karbach, 1992; Wasserman *et al.*, 1992; Fullmer, 1992). The effects of vitamin D_3 on calcium absorption via these pathways were established during investigations by Sallis (1962) and Bronner (1991). To maintain the calcium level of plasma at 10 mg/100 ml, calcium must be pumped from the intestinal lumen, the renal tubular fluid and the bone fluid compartment to the extracellular fluid (Deluca, 1980). The net absorption of calcium from the gut to the extracellular fluid is approximately 150-200 mg/day in human (Mundy, 1990). This rate is dependent on calcium intake from the diet and the circulating concentration of 1,25-dihydroxyvitamin D_3 and other dietary components (lactose, glucose, amino acid and phosphate) which may serve to alter calcium absorption from the intestine (Deluca,

1980; Panus *et al.*, 1983; Mundy, 1990). Calcium uptake from the proximal two-thirds of the small intestine has been found to be most rapid within 4 hours of feeding in chickens (Harrison, 1951; cited by Sallis, 1962). The requirement of calcium during laying (eggs in uterus) can be provided by increased intestinal absorption and bone mobilisation (Johnson, 1986). Isotopic studies have determined the influx and efflux of ^{45}Ca across the shell gland: at rest, influx and efflux are equal but while the egg is being calcified the influx is 2.4 times greater than the efflux to uterus (Simkiss and Tayler, 1971). The major part of this calcium must be provided by the small intestine.

Calcium absorption from the gut lumen is dependent on two mechanisms: an active process and a process of facilitated diffusion (Wasserman *et al.*, 1992). The transcellular route is dominant in the proximal intestine (Bronner *et al.*, 1986). Transcellular movement involves three stages: (1) entry across the brush border membrane of the enterocyte, (2) intracellular movement and (3) extrusion across the basolateral membrane (Bronner, 1992) (Figure 1.7). The primary regulator of intestinal calcium transport is the vitamin D endocrine system, specifically the hormone, 1, 25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), (Brody, 1994). There is evidence that supports multiple roles for this hormone in the three steps transport process (Weiser *et al.*, 1981; Roche *et al.*, 1986; Miller *et al.*, 1981 cited by fuller, 1992). Vitamin D_3 may modulate to opening of calcium channels, synthesis of the protein which binds with calcium in the process of calcium transport and an increase of Ca ATPase activity (Schiffi and Binswager, 1980; Ghijsen and Van Os, 1982).

1.2.6.3. Transcellular calcium transport

The entry of calcium into the intestinal cell of the brush border membrane requires no metabolic energy, as calcium moves down a steep electrochemical gradient, because the calcium concentration is much higher outside than inside the cell (Fullmer, 1992). Calcium entry might be influenced by calcium channels, but there is little direct evidence for this situation (Fullmer, 1992).

Over the years, a number of factors have been recognised in the process of calcium entry. A number of these will now be described briefly. Alkaline phosphatase known to be present in the brush border region of intestine epithelial cells, hydrolyses a number of organics (Ghijsen *et al.*, 1980) The activity of this enzyme is reduced in vitamin D deficient rats and chicks as compared with normal control animals (Shimura and Wasserman, 1984).

Two specific calcium binding proteins have been identified in brush border (Shimura and Wasserman, 1984). It was proposed that calcium-binding complex (CaBC) which is isolated in rat brush border membranes and intestinal membrane calcium-binding protein (IMCal) might function to mediate the transit of calcium from the lumen, across the brush border membrane, to the cytosol (Fullmer, 1992). In rats, calcium binding activity was generally well correlated with known features of calcium transport including dietary calcium level, age, intestinal distribution and vitamin D repletion (Shimura and Wasserman, 1984). Bikle *et al.*, (1984) reported that calmodulin which may mediate calcium flux across the intestinal brush border membrane in responsive* to $1,25(OH)_2D_3$ administration. Vitamin D_3 can also increase some lipids such as arachidonic* in the brush border membrane (Matsumato

et al., 1981). This response to calcium transport and so-called "liponomic theory" has been detected in the brush border membrane (Matsumato *et al.*, 1981; Brasitus *et al.*, 1986).

Inside the cell, calcium must move through the cytoplasm and intracellular movement of calcium needs a calcium carrier (Bronner *et al.*, 1986). Several carriers have been proposed for intracellular calcium transport: Calmodulin levels has a major role in making calcium binding proteins, like calcium binding protein (CaBP) and serve to buffer intracellular calcium transport. In addition, calmodulin, in response to 1,25$(OH)_2D_3$ may serve to mediate the entry of calcium into epithelial cells (Bikle *et al.*, 1984). It has been suggested that calmodulin directly effects the calcium pump and it has also been indicated that fifty percent of duodenal calcium pump activity is related to calmodulin (Thomasset *et al.*, 1981; Ghijsen and Van Os, 1982). Data from electron microscopy have suggested carrier roles for mitochondria, golgi, endoplasmic reticulum and in particular the endosomal-lysosomes pathway in intercellular calcium transport. Microtubules, cytoskeletal elements along which vesicles move, have been found (Wanner and Coleman, 1975; Davis *et al.*, 1979; Rubinoff and Nellans, 1985) to contain 1.25$(OH)_2D_3$ regulated protein and also calbindin D_{28k} in chick intestine. These may facilitate the intracellular movement of calcium in the cytoplasm (Nemere, 1990). Thus the vitamin D_3 role as a regulator in CaBP synthesis in the intracellular calcium transport is very important because this vitamin can influence calbindin gene expression (calbindin D28k) in the chicken and therefore increase intracellular calcium transport. In the presence of vitamin D_3 intracellular calcium transport is faster than when vitamin D_3 is deficient (Wasserman *et al.*, 1992; Wilson and Lawson, 1980).

Calcium transport and extrusion processes at the basolateral membrane are related to the ATP-dependent Ca^{2+} pump, a Na^{+}/Ca^{2+} exchanger whose operation is dependent on a transmembrane Na^{+} gradient maintained by the Na^{+}/K^{+} pump, Ca^{2+} channels and exocytosis of Ca^{2+} containing vesicles (Walter and Weiser, 1987; Ambrecht *et al.*, 1988; Hamaidan *et al.*, 1989; Takito *et al.*, 1990). The existence of an energy-dependent active Ca^{2+} transport process in rat intestinal epithelium was first shown by Schachter and Rosen (1959). They found that the chick basolateral membrane contain an ATP-dependent Ca^{2+} uptake system.

The ATP-dependent Ca^{2+} transport by basolateral membrane vesicles has been positively correlated with intestine Ca^{2+} absorption as a function of animal age, intestinal segment and crypt-to-villus axis (Van Corven *et al.*, 1986; Walter and Weiser, 1987; Ambrecht *et al.*, 1988; Ghishan *et al.*, 1988). These observations have been interpreted as evidence that the basolateral calcium pump plays a major role in the active intestinal transcellular Ca^{2+} absorption. Also the properties and behaviour of the ATP-dependent Ca^{2+} pump of chick basolateral membranes have been investigated by Takito *et al.* (1990).

The cellular localisation of the Ca^{2+} pump was visualised by Immunohistochemical localisation, using a monoclonal antibody against the human erythrocyte plasma membrane Ca^{2+} pump (Niggli *et al.*, 1979). Antibody binding occurred primarily along the basolateral membrane of the intestinal cell (Wasserman *et al.*, 1992). Broke *et al.* (1990) demonstrated similar localisation of the calcium pump in the rat intestine. These immunohistochemical experiments with rat and chick intestine provide visual support for the Ca^{2+} pump with the basolateral membrane.

The stimulatory effect of $1,25(OH)_2D_3$ on the ATP-dependent Ca^{2+} uptake by intestinal basolateral membrane vesicles has been demonstrated by several groups (Freeman *et al.*, 1977; Walter and Weiser, 1987; Favus *et al.*, 1989; Takito *et al.* 1990; Wasserman *et al.*, 1992). Western blot analysis of intestine mucosa, using a monoclonal antibody produced against the erythrocyte Ca^{2+} pump, has indicated that the number of pump units is increased by 1,25-dihydroxycholecalciferol (Wasserman *et al.*, 1992). The possible involvement of calbindin D_{28k} as a direct stimulator of the Ca^{2+} pump was determined by Broke *et al.* (1987); Suzuki and Kono, (1980) therefore by increasing the number of calcium pump, calcium transport will be increased from the basolateral membrane. Calmodulin and calbindin as Ca^{2+} pump activators have a significant role (Carafoli, 1991; Ghijsen *et al.* 1986). Both the Ca^{2+} affinity of the pump and the maximal transport rate of calcium are increased by calmodulin (Roche *et al.*, 1986; Moncrief *et al.*, 1990). A question of interest is whether there is sufficient Ca^{2+} transporting activity associated with the basolateral Ca^{2+} pump to account for the active transport of Ca^{2+} across the intestinal epithelium. It has been calculated that the activity of the calcium pump is seven times in excess of what was necessary to accommodate intestinal active Ca^{2+} transport (Bronner *et al.*, 1986). In the same report, (Bronner *et al.*, 1986) using other assumptions, suggested that the Ca^{2+} pump activity might be in excess by a factor of about three in the cholecalciferol-replete rat.

Sodium-calcium exchange activity in plasma membrane vesicles is related to the Na^+/Ca^{2+} exchange system which is a carrier-mediate transport process for the movement of calcium ions across the membrane. The movement of Na^+ ions is

directly coupled in the opposite direction to that of calcium (Van Os, 1987). The exchange system is electrogenic with a stoichiometry in most cells of 3 Na^+ per Ca^{2+} (Van Os, 1987). The major physiological role of exchange system is to pump calcium out of the cell, using the inwardly directed electrochemical gradient for Na^+, maintained by the Na/K-ATPase as an energy source. It was reported that the transport capacity of calcium by Na^+/Ca^{2+} exchange was about 20% of ATP-dependent Ca^{2+} pump activity (Ghijsen, 1983). Although, increasing the NaCl in the drinking water of laying hens can increase the plasma calcium and phosphorous concentration, in contrast to this, shell calcium and shell thickness were reduced, because the activity of carbonic anhydrase can be reduced by NaCl in the shell gland mucosa (Balnave, 1993). This limits the provision of bicarbonate and the dependent calcium to the lumen of the shell gland (Balnave, 1993; Pourreza *et al.*, 1994).
Calcium channel is the other way for transport in the basolateral membrane. Permeability of the plasma membrane increases by the selective opening of Ca^{2+}channel allowing calcium to pass from the basolateral membrane along its electrochemical gradient. The gating of these channels is regulated by changes in membrane potential (Nilius *et al.*, 1985; Matteson and Armstrong, 1986). The identification of calcium channels in intestinal epithelial cells is important to our understanding of the regulation of cytosolic calcium concentration, Na and Cl transport (Hamaidan *et al.*, 1989).

Histological and electron microprobe analysis of the intestinal epithelium provides evidence that suggests that $Ca^{2+}$is transported through the cytosol in vacuoles. At the basolateral membrane vesicular Ca^{2+} is released into the lamina propria by exocytosis (Jande and Brewer, 1974; Wanner and Coleman, 1975).

1.2.6.4. Paracellular calcium transport

The paracellular transfer of calcium occurs when calcium concentration in the intestinal lumen is sufficiently high (Dostal and Toverud, 1984). The paracellular pathway consists of three successive structure: (1) the tight junction, (2) mediate junction and (3) basolateral space (Dostal and Toverud, 1984). It has been found that the rate of calcium movement through the region of the tight junction is far slower than would be expected on the basis of simple diffusion. Hence the structure of the junction, with its compressed protein-lipid space, must hinder fluid and calcium movement appreciably. Hyperosmolar solutions, regardless of their chemical nature, can cause the rate of passive calcium transport to double or triple (Pansu *et al.*, 1976). This presumably occurs because water moving into the hyperosmolar space causes the tissue to expand and the junctions to widen. This, in turn, must result from a modification of the cytoskeleton of the cell walls lining the junctions (Pansu *et al.*, 1976).

It has been proposed (Pappenheimer, 1987) that at least some amino acids cause contraction of the cytoskeleton of the cells lining the junction, thereby causing increased calcium flow. Many years ago, it was reported (Wasserman *et al.*, 1956) that feeding l-lysine caused calcium absorption to be increased. Also it has been found that $1,25(OH)_2D_3$ increased calcium permeability by changing the phospholipid composition of the brush border membrane (Rasmussen *et al.*, 1982). The recycling of water flow may induce secretion of calcium across the tight junction in a direction opposite to transepithelial fluid absorption (Ussing and Johansen, 1969). It is conceivable that the vitamin may also change the chemical structure of the junctional complex and thereby increase paracellular calcium flux (Karbach, 1992).

1.2.6.5. Regulated calcium transport in intestine and shell gland

All processes consist of paracellular and transcellular calcium transport with different aspects of the cell transporting calcium from the cell to the bloodstream. The concentrations of serum, calcium ionised and bound, in the laying hens blood are 6.6 and 27.45% respectively (Scott and Zeigler, 1963). Calcium required by the uterus is carried by the bloodstream from the intestine. Blood calcium can be maintained constant by parathyroid hormone and vitamin D_3 activity. These hormones support calcium levels in the shell glands of the uterus to assist eggshell quality. Calcium demand is increased during the shell formation (Simkiss and Tayler, 1971; Eastin and Spaziani, 1978). In fact, it is still possible to detect which factors can affect calcium secretion in the uterus and egg shell quality.

An investigation into the distribution of ^{45}Ca between the subcellular organelles of the active and inactive shell gland have shown that the mitochondria of shell glands accumulate calcium to a much greater extent than do those of the liver (Simkiss and Tayler, 1971). These findings indicate that the epithelial cells of the shell gland actively move calcium ions from the blood stream to the lumen of the oviduct and carbonic anhydrase enzyme has a significant role in this transfer (Mongin, 1968; Simkiss and Tayler, 1971). Calcium ions are temporarily stored in the mitochondria when calcification is not occurring but are moved out and transported out of cell via the endoplasmic reticulum (microsomal fraction) during shell formation (Simkiss and Tayler, 1971). CaBP (calbindin D_{28k}) play an important role not only in the intestine but also in the uterus. In both organs an increase in calbindin D_{28k} is coupled with an increase in calcium transport (Bar *et al.*, 1978). The concentrations of calbindin and calbindin mRNA are increased at the onset of egg production in the intestine and

shell gland in the uterus (Bar *et al.* 1990). This increase is not only dependent on vitamin D_3 but is associated with the calcification of the first egg (Bar *et al.*, 1990). The synthesis of ATP in the mitochondria from ADP and (Pi) is coupled to movement of protons down their electric and chemical gradients. This resembles the reaction catalysed by the Ca^{2+} ATPase in which the movement of Ca^{2+} down its concentration gradient powers the synthesis of ATP from ADP and P_i (Darnell *et al.*, 1990). Movement of calcium across the shell gland may occur by both diffusion and active transport (johnson,1986). A brief explanation of transcellular calcium transport is shown in Fig. 1. 6, (Favus, 1991).

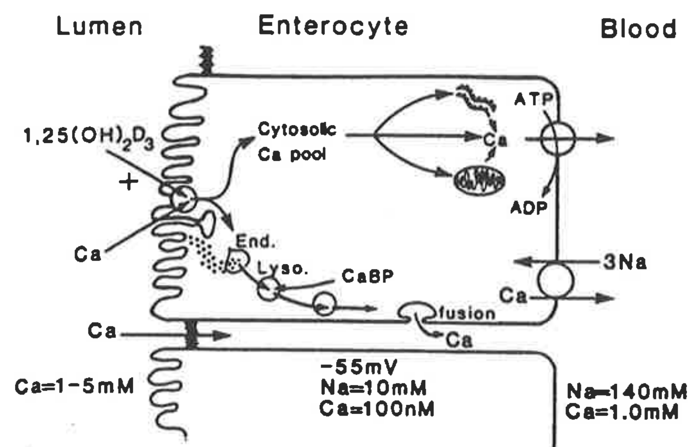


Figure.1.6: Potential pathways and mechanism for calcium transport across intestinal epithelium. From Favus and Tembe, 1992.

1.2.6.6. Structure and function of calcium binding protein.

CaBP is the superfamily of EF-hand helix-loop-helix as the major components in the molecular shape. Thirty-nine subfamilies of calcium binding proteins have been

described and embedded by more than 250 proteins (Celio *et al.* 1996). The common structure has been discovered for all these proteins, which consists of an α-helix (residues 1-10), a loop surrounding calcium ions (residues 10-20) and a second α-helix (residues 19-29) which is presented almost at right angles to the first (Celio *et al.*, 1996) (Fig. 1.7) Calcium ion is furnished by 7 oxygen ligands that are linked by acidic residues in the side chains and also carbonyl groups of the peptide backbone with bridging water molecules. The three dimensional shape of EF-hand structure can be illustrated by considering the right hand, the index finger representing the E-helix, the bent middle finger showing Ca^{2+} binding loop and the F-helix depicted by the thumb (Fig. 1.8). The Ca^{2+}-binding loop includes a β-sheet which is very important for pairing two EF-hands into the tandem domain as well as for the function of Ca^{2+}-binding sites (Celio *et al.*, 1996).

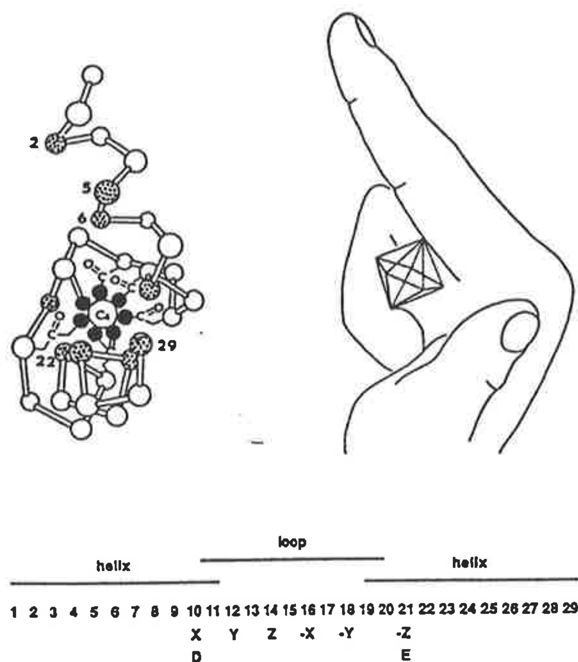


Figure 1.7: The EF-hand Ca^{2+}-binding protein with two α-helices, loop and calcium which is co-ordinated by seven oxygen ligands. Modified from Moncrife *et al.*, 1990, cited by Celio, 1996.

Calcium binding proteins contain calbindin D_{28k} which is detected in mammals and avian species and generally a large class of eukaryotic cytosolic proteins (Akerfeldt *et al.* 1996) or calbindin D_{9k} that are expressed particularly in mammalian tissues. Calbindin D_{28k} consists of 261 (mammalian) or 262 (avian) amino acids (Hunziker and Bendik, 1996). Calbindin D_{28k} was first observed as protein in the duodenal mucosa of chicken and induced by vitamin D (Waserman and Taylor, 1966, cited by, Hunziker and Bendik, 1996).

Calcium absorption in the small intestine responds to vitamin D activity in this organ (Hunziker and Bendik, 1996). Much attention has been made to the molecular action of vitamin D in the synthesis of protein or proteins involved in calcium absorption (Wasserman *et al.*,1978). The intestine is the primary target organ of vitamin D in expression of CaBP. Subsequent studies have demonstrated its presence along the entire length of the small intestine (Taylor and Wasserman, 1970b, cited by Wasserman *et al.*, 1978). CaBP Expressed in the cytoplasm and nucleus of the cells in the small intestine (Jande *et al.*, 1981). It is already well documented that calcium absorption in the jejunum of the small intestine of laying hens increases during shell calcification (Wu *et al.*, 1993). The shell gland of laying hens also contains calbindin D_{28k} during shell formation and is involved in calcium translocation to the egg shell (Wasserman *et al.*, 1978; Berry and Barke, 1990). CaBP has been observed in different species of animals and different organelle tissues such as small intestine, liver, kidney, brain and blood (Jande *et al.*, 1981).

Immunohistochemical procedures have been employed to identify CaBP localisation in the intestinal mucosa as well as in other tissue (Jande *et al.*, 1981). Several

investigations have indicated that CaBP was present in goblet cells of the brush border in the cytoplasm and nucleus of absorptive epithelial cells in the mucosa of chicken small intestine (Taylor and McIntoch, 1977; Noda *et al.*, 1978, cited by Jande *et al.*, 1981). Similar results noted that the rat contained CaBP in the intestinal absorptive cells but not in goblet cells (Marche *et al.*, 1979, cited by Jande *et al.*, 1981).

The concentration of calbindin and its mRNA in the duodenum, jejunum and shell gland of laying hens was greatly increased during the onset of lay, particularly during the period of calcification of the first egg (Bar *et al.*, 1990; Wu *et al.*, 1993). The onset of egg production is associated with high demand of $1,25(OH)_2D_3$, this increase may respond to the indirect effects of estrogen on the kidney. Calbindin synthesis in the small intestine and shell gland can be induced by the other factors influencing transcription as well as post-transcriptional stages (Bar *et al.*, 1990). Estrogen can cause changes in cholecalciferol metabolism, medullary bone formation as well as changes in plasma-bound calcium (Bar and Hurwitz, 1979). It is well known that, alter the calcium metabolism of birds is a response to endogenous estrogen (Hurwitz, 1989). The result is either a direct initiation of hydroxylase system in the kidney, or a primary effect on medullary bone formation. This could be followed by an increases in $1,25(OH)_2D_3$, CaBP as well as an increase in calcium absorption in the small intestine of laying hens during eggshell formation (Bar and Hurwitz, 1979). The possibility of the direct action of estrogen on calbindin D_{28k} synthesis can be affected in this aspect.

1.2.6.7. Intestinal enzyme activity

The CaPB (calbindin D_{28k}) is not the only factor in calcium absorption, therefore other factors must be involved in calcium absorption. Such factors include, protein plasma membrane and enzyme activity (Bradford, 1976; Nys and Laage, 1984).

The activities of membrane-bound enzymes alkaline phosphatase, Ca and Mg-ATPase are increased by $1,25(OH)_2D_3$ (Nys and Laage, 1984). They are also involved calcium absorption process in the small intestine (Lane and Lawson, 1978; Nys and Laage, 1984). Jejunal ATPase activity as well as egg shell quality can be reduced by the lack of vitamin D_3 supplementation in laying hen diets (Grunder and Tsang, 1984).

ATPase activities in active transport across biological membranes have been stimulated by Na^+/K^+. The activities of intestinal epithelial transport cells are not only dependent on Na^+ and K^+ but certain sugars and amino acids are essential for this type of transport. An interaction of sugar, amino acids and Na have been found in the intestine in several species (Quigley and Gotterer, 1969). Sodium as a mediator of common carrier could be linked directly or indirectly to Na^+/K^+ ATPase. In addition, it seems in epithelial plasma membranes, Na^+/Ca exchange has a basic role, since Na changes Ca transport in a direct way as well as indirect action on ATP-dependent Ca^{2+} (Van Os, 1987).

Recent evidence has indicated that enterocyte calcium influx was mediated by the cycle AMP which stimulated by estrogen, because the present estrogen receptor in intestinal mucosa of duodenum has been identified in rat (Picotto *et al.*, 1996). This suggested that estrogen participates in hormonal regulation of calcium transport in the intestine through the genomic and non-genomic pathways. It is well established

that $1,25(OH)_2D_3$ can effect on calcium transport via genomic (induce calcium binding protein synthesis) and non-genomic (activity of voltage) in calcium channel. Piccotto *et al.* (1996) have reported that the 17β-estradiol effect on enterocytes calcium uptake could be elucidated by the presence of a signalling estrogen receptor on the cell surface of membrane. The effect of nuclear estrogen receptor is not likely, since the compound ICI 182 780 as (an antiestrogen) did not abolish the effect of 17β-estradiol on calcium transport (Picotto *et al.*, 1996).

It has been demonstrated that there is a link between the concentration of plasma estrogen and activities of Ca, Mg, Ca/Mg ATPase in chicken shell gland and this pattern was more significant in laying hens rather than non-layers (Qin *et al.*, 1993). Intestinal activity of ATPase enzymes corresponded with activity of these enzymes in shell glands and also concentration plasma estrogen (Nys and Laage, 1984; Qin and Klandorf, 1993). ATPase activity in total shell gland homogenate was significantly greater in hens with strong eggshells rather than those with weak shells (Castaldo and Maurice, 1989). The considerations have shown that Ca^{2+}-dependent or activated Ca ATPase may be involved in active transport in the uterus and duodenum (Watanabe *et al.*, 1987).

1.2.6.8. Role of hormones

Calcium transport across bone, kidney and gut is regulated by hormones such as parathyroid hormone, 1,25 dihydroxyvitamin D_3 and estrogen and their interactions within extracellular fluid (Deluca, 1980; Feher, 1983 and Nemere *et al.*, 1984).

Calcium absorption also is controlled by hormones, these includes sex steroids which

act to promote medullary bone formation and stimulate calcium transport in the intestine and uterus by vitamin D metabolites (Taylor *et al.*, 1971; Nemere and Norman, 1991). Calbindin and calbindin mRNA in the intestine and uterus can be increased by interaction between steroid hormones such as estrogen and 1,25$(OH)_2D_3$ (Bar *et al.*, 1990). The amount of the physiologically active metabolite, 1,25$(OH)_2D_3$ increases slightly as immature hens become sexually mature and this difference is greater at the onset of egg laying (Wu et al., 1994). Calcium transport increases during this period of development (Bar *et al.*, 1978; Wu *et a*l., 1993). 1,25$(OH)_2D_3$ leads the final expression of calbindin which involves the binding of 1,25$(OH)_2D_3$ receptor to nuclear DNA. It also increases calcium transport in the intestine and uterus. A similar condition of calbindin in the rat uterus has been observed through the nuclear binding of an estrogen to estrogen receptor complex (Delorme *et al.*, 1983; Bruns *et al.,* 1988; Bar *et al.*, 1990). Wu *et al.* (1994) reported on the probable interaction of estrogen with its intestinal receptor in mature layer hens as well as the role of a vitamin D receptor in promoting gene transcription. Little is known of the mechanism involved. On the other hand several dietary factors such as 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane (DDT), aluminium and 1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene (DDE) can inhibit reproductive capacity and reduced egg shell quality (Nyholm, 1981).

Parathyroid hormone (PTH) is secreted by the major cells of the parathyroid glands. This hormone consist of an 84-amino acid single chain polypeptide. PTH secretion is induced by a fall in plasma calcium in the parathyroid gland. This gland plays a central role in the regulation of plasma 1, 25-dihdroxyvitamin D_3 as at low calcium concentrations, the hormone stimulates bone reabsorption and could be as an

activator for converting of $25(OH)_2 D_3$ to $1,25(OH)_2D_3$ by stimulatory hydroxylase enzyme in the kidney. Finally, there is an increase in the amount of calcium delivered to ~~into~~ the circulation. The major effect of PTH on the kidney is to increase distal tubular reabsorption of calcium (Mcsheehy *et al.*, 1986). Bloodstream calcium ions can come from three sources: The diet, the skeleton and glomerular filtrate of the kidney. On the other hand, renal tubular reabsorption of phosphate and bicarbonate are prevented by PTH in the proximal tubules. (Royle *et al.*, 1972; Deluca, 1980; Nemere *et al.*, 1984; Mundy, 1990).

Vitamin D metabolites are steroid-like compounds derived from plant ergosterol ingested in the diet or synthesised in the skin by exposure of epidermal cells to ultraviolet light, such as 7-dihydrocholesterol to vitamin D_3(Deluca, 1980 and Brommage *et al.*, 1984). Vitamin D_3 can be converted to 25- hydroxy vitamin D_3 (25-OH-D3) by liver microsomes and also in the final stage, can be changed to 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) by kidney mitochondria (Deluca, 1980; Takahashi *et al.,* 1982; Mundy, 1990). This metabolite (1,25(OH)2 D3) regulates and synthesises calcium-binding protein (CaBP) in the intestine and uterus plays a major role in calcium transport (Shiwki *et al.*,. 1982; Johnson, 1986; Bar *et al.*, 1987). Shell quality is also improved by $1,25(OH)_2D_3$ (Bar *et al.*, 1988) . Shell formation and the mineralisation process in bone are dependent on the major effect of 1, 25-dihydroxyvitamin D_3 on the gut and bone (Deluca, 1980 ; Feher, 1983 and Mundy, 1990).

Recent *in vitro* studies have shown that regulation of the renal and intestinal calbindins in the post transcriptional mechanisms is carried out by 1,25(OH)2D3. In

addition, calbindin mRNA also is regulated by the vitamin D hormone at the transcriptional and post transcriptional level. Stabilisation of transcribed RNA may be an important mechanism in the regulation of calbindin gene expression by $1,25(OH)_2D_3$ (Wasserman, 1981; Bar *et al.*, 1990; Kjama *et al.*, 1991; Nys *et al.*, 1992; Wu *et al.*, 1993).

Results from several new studies indicate that although some vitamin D receptor (VDR) is located in the nucleus, the majority of VDR exists in the cytosol. These receptors are able to link with numerous hormones and also inducing estrogen (Wack, 1994). Interaction may induce calcium transport in the intestine and uterus (Boyle *et al.*, 1972 and Deluca, 1990). These factors, therefore may have an indirect effect on egg shell quality because they may affect calcium transport in the both intestine and uterus (Bar *et al.*, 1990).

The physiological role of estrogen as a female sex hormone is known well. This hormone not only promotes and develop ovary and oviduct growth and function but it has been indicated to effect the composition of the blood and metabolism the egg precursors (Turner, 1948) It has been demonstrated that the sexual maturity in the birds, estrogen secretion, the metabolism of fat and calcium and serum calcium are increased to form the lipid of the yolk and the calcium of the egg shell (Bucker *et al.*, 1930 and Macowan, 1932 cited by Turner, 1948). Feeding, tablets and injection as sources of the estrogen at the optimal levels may promote fat and calcium metabolism in the laying hens and therefore make possible more sustained egg production (Turner, 1948; Pike*et al.*, 1978 and Baksi *et al.*, 1982).

1.2.7. Estrogenic diets (Phytoestrogens)

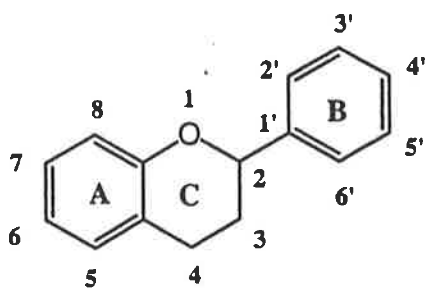
Phytoestrogens (plant estrogen) have been found in a wide variety of plants as well as in cereals, vegetables, alfalfa clovers, and fruit (Petterson and Kiessling, 1984; Knight and Elden, 1996). Legume seeds have been shown to have marked estrogenic activity (Franke *et al.* 1998). In both flowering and non-flowering plant tissues, they are conjugated to sugars and can be isolated, although a non-conjugated form (aglycones) has also been observed in nature (Miksicek, 1993). One of the basic roles of these materials in plants is to provide the pigmentation of the petals of flowers (Miksicek, 1993). Different methods of extracting phytoestrogens have been reported. Estrogenic activity of forage is reduced by drying (Bickoff, 1959; Petterson and Kiessling, 1984). It is well established that the quantity of phytoestrogen in plants is dependent on age, growth stage and degree of environmental stress such as dry conditions (Lien and Cain, 1985). Many phytoestrogenic chemicals have an aromatic structure. These include a number of the bioflavonoid compounds (flavones, isoflavones, flavanones), lignane, coumestrol from legumes, and zearalenone a mycotoxin derived from fungal moulds. While bioflavonoid compounds are widespread in plant and foods, genistein and daidzein with significant estrogenic activity have been recognised in this class of phytoestrogen, with the largest concentration in soybean (Reinli and Block, 1996; Baghurst, 1997). Formononetin is found in clovers and lignans. It has a 2,3 dibenzyebutane structure and is widespread in plant foods. Lignans exist as blocks in the formation of lignin in plant cell walls (Knight and Elden, 1996). It is known that mammalian have the common names of enterolactone and enterodiol. These compounds have been identified in human urine, serum, faeces, semen and also in cow's milk (Knight and Elden, 1996). The structure of some common phytoestrogen compounds are shown in the Figure 1.8, (Wall and Samman, 1997).

More than 300 plants have been found to cause estrogenic responses in animals (Franke *et al.* 1998). Infertility problems in livestock have been found to be caused by their feeding on forage plants such as clover, due to high levels of isoflavones (Franke *et al.*, 1998). Reduction in the reproductive performance of sheep and cows consuming estrogenic plant silage have been described (Petterson and Kiessling, 1984). The isoflavone formononetin and its metabolites daidzein and equol have been found to be responsible for the infertility syndrome "clover disease" (Welshone *et al.*, 1990). As a result, dietary estrogen can cause hyperestrogenism and clover disease causing reproductive problems of economic extent in the US and Australia, as well as in other countries (Welshone *et al.*, 1990).

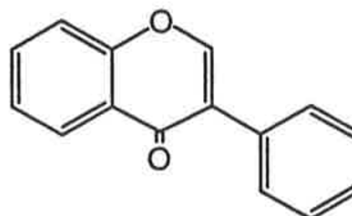
There is little information regarding phytoestrogens effects in birds, particularly on the reproductive performance of laying hens. Phytoestrogens in green foliagecan inhibit reproduction in California quail during dry years (Lien and Cain, 1987). Delay in the onset of egg production was observed in California quail when clover was added to the normal breeder diet as a high phytoestrogenic source (Lien and Cain, 1987). No impairment was found in reproductive system of Bobwhite hens by consuming up to 1 mg/day of biochanin-A, a phytoestrogen in natural feedstuffs (Lien and Cain, 1987).

Martin *et al.* (1978) have shown that estrogen receptors complexed to phytoestrogens (genistein, coumestrol or zearlenol) are processed in the nucleus at about the same rate of estradiol binding receptor in human. This demonstrated that phytoestrogen not only binds to the estrogen receptor but may have a biologically effect on the reproductive system (Martin *et al.*, 1978; Lien *et al.*, 1985). The phytoestrogen

Generic structure

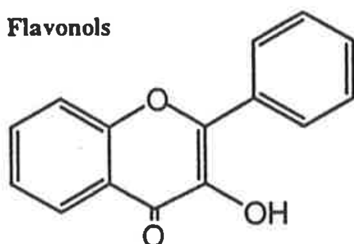


Isoflavones



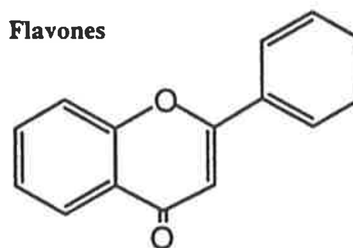
	5	6	7	4'
Genistein	OH	-	OH	OH
Daidzein	-	-	OH	OH
Glycitein	-	O-Me	OH	OH

Flavonols



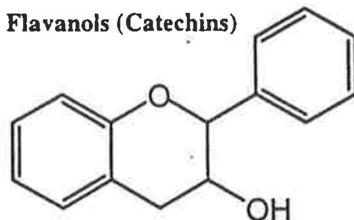
	5	7	3'	4'	5'
Myricetin	OH	OH	OH	OH	OH
Quercetin	OH	OH	OH	OH	-
Kaempferol	OH	OH	-	OH	-

Flavones



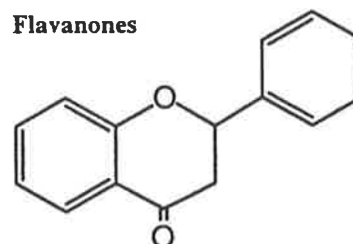
	5	7	3'	4'
Luteolin	OH	OH	OH	OH
Apigenin	OH	OH	-	OH

Flavanols (Catechins)



	5	7	3'	4'	5'
(+) Catechin	OH	OH	OH	OH	-
(-) Epicatechin *(cis)*	OH	OH	OH	OH	-
(-) Epigallocatechin	OH	OH	OH	OH	OH

Flavanones



	5	7	3'	4'
Naringenin	OH	OH	-	OH
Hesperetin	OH	OH	OH	O-Me

Figure 1.8: Stucture of the major classes of phytoestrogen (flavonoinds), position of hydroxy group on A and B rings are listed for selected examples within each class. From Wall and Samman (1997).

content of the diet of laying hens may play a priority role in the synthesis of protein, associated with calcium transport, by affecting the transcription stage and binding to DNA, consequently affecting egg production and eggshell quality. Genistein and daidzien have long been known to be the major constituents of phytoestrogens in soybean (Setchell and Welsh, 1987; Wang *et al.*, 1990; Kudou *et al.*, 1991; Coward *et al.*, 1993) a plant protein source in poultry diets. Further investigations are needed to be carried out regarding the effect of these sources of phytoestrogens (genistein and daidzien) and their amount that can be permitted in commercial laying hens diets.

1.2.8. Aim and objectives

High demands of calcium in laying hens compared with the other domestic animals (2 g calcium for each eggshell formation) is a critical topic. It is well doucumented that the calcium requirement of laying hens increases during sexual maturity and the onset of lay in pre laying hens (Wu *et al.*, 1994). In addition vitamin D_3 ($1,25(OH)_2D_3$) is strongly associated with calcium transport in the intestine of laying hens. Although the production of the vitamin D_3 metabolite ($1,25(OH)_2D_3$) is increased by the indirect effect of estrogen on production of 1-hydroxylase enzyme in the kidney. There is a doubt for direct effect of estrogen on calcium transport across the small intestine in pre-laying hens.

This study will examine the effect of estrogen and phytoestrogen on calcium retention in the intestine of pre-laying hens, especially in the jejunum as this needs to be clarified. Interaction between estrogen and phytoestrogen is still unclear and needs further investigations. Also these interactions may effect calbindin synthesis in the intestine. Investigations will be conducted to determine the effect of the optimum levels of synthetic estrogen and phytoestrogen, on calcium absorption. Finally, these

findings should explain the mechanism of calcium retention in laying hens which is affected by estrogen and phytoestrogen and will lead to consideration of egg shell quality and egg production.

Although much research has been conducted into calcium metabolism of laying hens, only limited work has been carried out into the interaction between calcium and estrogen, or phytoestrogens (a major component in soybean meal used in commercial laying hens diets) may play specific role in egg production and shell quality. Enzyme activity, expression of calcium binding protein in regard to egg production and eggshell quality also will be assessed in this study.

Finally the aim of this study is to determine to what degree calcium retention is under the control of nutritional, and hormonal (estrogen and phytoestrogen) treatments in pre-laying hens. The full objectives of the research will determine:

1. The effect of different levels of estrogen and calcium on calcium retention in the intestine of pre-and point-lay hens.

2. The relationship between calcium retention, calbindin, and estrogen in the intestine of different strains of pre-point laying hens.

3. The interaction between alkaline phosphatase and ATPases enzymes, estrogen, calcium and strains on egg and eggshell quality.

4. The influence of different levels of estrogen, phytoestrogen and their interaction on the calcium retention, calbindin D_{28k} expression in intestine on egg production and eggshell characteristics at pre and point lay hens.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

"The resistance of common eggshell to pressure applied from the inside of the shell is more than most automobile tyres" therefore it needs to be measured by good methodologies.

(Etches, 1996)

2.1. Introduction

Most of the experimental procedures and methodologies referred to in this thesis have not been used in investigations with poultry previously, but have been reported in studies with other species. This chapter describes materials and procedures common to more than one experiment which were carried out in this research project. Other specific materials and methods of individual experiments are provided in each chapter.

2.2. Animals

Pre-laying Leghorn and Tegel pullets (10-18 weeks of age) were used in this study. In addition, to a limit extent, Isa-brown hens were used also. All birds were kept in individual cages under the same rearing conditions. Each bird was provided with individual feed and water, and was adapted to housing conditions for 3 days before each experiment. Ethics clearance for animal experimentation in this study was approved by the University of Adelaide according to NH and MRC guidelines.

2.3. Growth assessment

Growth was assessed in terms of weight gain (WG) by difference of initial (IBW) and final body weights (FBW) measured in kilograms. At the end of each experiment hens were euthanased, their intestine, gizzard, liver, oviduct and shell glands (g) were removed rapidly and the wet weights of each recorded. The intestine and gizzard were weighed with digesta and empty to determined their growth and capacity. The growth of liver, oviduct and shell gland (without its egg) were also measured. The weight and growth of these organs were calculated per 100 g body weight.

2.4. Feed and feed analysis

Diet ingredients, feed composition and preparation of feeds used in all experiments in this study were of commercial quality formulated to the National Research Council standard (NRC, 1994). Feed was stored under cool (14° C) and dry conditions.

2.4.1. Dry matter measurement

During the experiment, feed and faecal samples were collected and weighed daily for 10 days. They were stored at - 20° C. After each experiment all samples of each hen (10 samples) were combined and two sub-samples removed. Dry matter (DM) of these sub- samples (feed and faecal) were determined by drying at 60°C in an oven for 24 hours using the following equation.

$$DM\% = \frac{Dry\ Samples\ (g)}{Wet\ Samples\ (g)}\ x\ 100$$ (McDonald *et al.*, 1995; Pond *et al.*, 1995).

The dry samples were grounded separately and stored in labelled plastic bag and kept in a cool and dry place for chemical analysis.

2.4.2. Energy measurement

Metabolizable energy of the feed, was calculated according to the methods of Australian Pig and Poultry Feed Composition (Evans, 1985), National Research Council (NRC, 1994), Banerjee (1992) and McDonald *et al.* (1995).

2.4.3. Protein measurements

Crude protein (CP) in dry ground feed and faecal samples was estimated from the

nitrogen content determined using a nitrogen analyser (Carloerbr, St 1500 b-E, 1983, using Kjeldahl method based on digestion and titration stages). CP was calculated by multiplying the N content by 6.25, using the assumption that protein contains 16 percent nitrogen (McDonald, *et al.* 1995).

2.4.4. Fibre and fat content of feed

The crude fibre (CF) and total fat (ether extract, EE) which effect energy content of feed, were measured by the methods of NRC (1994), Mcdonald *et al.* (1995) and Evans, (1985).

2.4.5. Minerals

The minerals calcium (Ca), phosphorous (P), magnesium (Mg), sodium (Na), potassium (K) in feed and faecal samples were measured using Inductively Coupled Plasma Spectrophotmetry (ICP), ARL, model 3580B analyser. The samples were ground in a suitable mill (1mm) to obtain a homogeneous mixture and then dried in an oven (80°C), and made free from any contamination from either soil (by removed them) or hand cream and cosmetics which may effect the results of analysis (using gloves). Eleven milliliters of an acid mixture ($HCLO_4$: HNO_3 , 1:10) was added to 0.6-0.8 g of samples and incubated overnight at room temperature. The laboratory standard or NIST (National Institute Standard and Technology) and blank pairs were used for each thirty samples to standardise the ICP machine.

2.4.6. Phytoestrogen identification and measurement

Phytoestrogens were extracted from 100 mg of feed in 10 ml of 80% methanol by

heating for 1 hr at 60° C. The mixture was centrifuged at 3000 rpm for 10 min., the supernatant decanted and dried under nitrogen. The residue was then resuspended in 1 ml 80% methanol plus 2 ml of glucosidase (ICN Biomedicals, Aurora, OH, USA) and incubated for 20 hr at 37° C. The final step was to extract the mixture with 12 ml diethyl ether which was then dried down under nitrogen and resuspended in 1 ml 80% methanol for analysis. The phytoestrogens, daidzein and genistein were isolated by high performance liquid chromatography (HPLC) based on the methods of Reinli and Block, (1996).

2.4.7. Feed intake and feed conversion ratio (FCR)

Daily feed intake (DFI) and feed conversion ratio (FCR) are two items that need to be determined in animal production. Daily feed intake (g/day) was measured daily (over a 10 days period) by weighing both feed offered and feed residuals at 8 am and 4 pm (g/day). The feed was prepared as a mash and given *ad libitum* to all pullets during the experiments. The other rearing conditions such as (temperature, humidity and lightning), which may effect feed intake were maintained constant in each and between experiments of this study.

Feed conversion ratio (FCR) was determined during all experiments of this study. FCR with respect to either egg production or body weight gain was calculated using the following equation:

$$FCR = \frac{Weight\ gain\ or\ egg\ production\ (g)}{Feed\ consumption\ (g)}$$ (McDonald *et al.*, 1995).

2.4.8. Nutrient retention

Retention of dry matter (DM), crude protein (CP), calcium (Ca), phosphorous (P),

sodium (Na), potassium (K) and magnesium (Mg) as factors influencing on calcium retention and absorption were calculated as follows:

$$Rétention = \frac{(Feed\ Nutrient\ x\ Dry\ matter - Faeces\ Nutrient\ x\ Faeces\ Dry\ matter)}{(Feed\ Nutrient\ x\ Dry\ matter)} x\ 100$$

(Banerjee, 1992; McDonald *et al.,* 1995; Pond *et al.*, 1995).

2.5. Egg production

The rate of egg production was determined by calculating the number of the eggs produced per hen during the period of production (days of egg production) expressed as a percentage. This was identified as an egg/hen/house and was calculated by:

$$Egg\ /\ hen\ /\ house = \frac{Number\ of\ eggs}{Number\ of\ hens\ days} x\ 100$$ (Etches, 1996).

2.5.1. Egg and egg shell characteristics

Eggs were collected every afternoon at 4 pm for 20 successive days and weighed fresh. Egg mass was calculated (g/day) during the period of egg production in experiments. The eggs were broken at the equator and the contents, except the shell membrane, were removed by cleaning with lukewarm water. The shell was then dried in an oven at 60° C for 12 hr, then cooled to room temperature and weighed (g). Shell thickness was measured at three points on the equator using a Mitutoyo Digimatic Outside Micrometer (model 1049) in µm. Eggshell weighed (g) and eggshell surface area were calculated using the equation ($S= kw^{0.67}$), where S = surface (cm^2), k, as a constant = 4.63 and W= egg weight (g) (Etches, 1996). Shell percentage was expressed as a percentage of egg weight. Shell weight in (mg/cm^2) was also calculated. The specific gravity (ESG) was determined by the equation:

$$ESG = \frac{EW}{0.9680\ x\ (EW - SW) + (0.4921\ x\ SW)}$$

EW, egg weight (g) and SW, Egg shell weight (g), (Harms *et al.,* 1990).

2.6. Sampling of small intestine

Intestinal samples (duodenum, jejunum, ileum) were removed rapidly from euthanased hens and placed in ice cold phosphate buffer saline, 130mM NaCl, 7mM Na_2HPO_4 and 3mM NaH_2PO_4 per litter distilled water (P.B.S, pH 7.4). One cm samples were fixed in Carnoy's fixative (60 ml ethyl alcohol, 30 ml chloroform, 10 ml glacial acetic acid) or 10% neutral buffer formalin (10 ml formaldehyde 40%, 90 ml distilled water, 0.35 g anhydrous sodium dihydrogen phosphate, and 0.65 g disodium hydrogen phosphate, pH, 7.4), Bancroft and Cook (1984). The remaining tissue from each region was snap frozen and stored at - 80° C for biochemical analysis.

Tissue sections of 0.5 cm were prepared from these samples and processed in cassettes (tissue-tek). Tissue processing was carried out in a Shadon Citadel Tissue processor using the following program. Samples were rinsed and then graded in 70% and 80% ethanol (for 1 hour each), twice in 95% ethanol (for 30 min and 1 h respectively) and also twice in absolute ethanol for 2 h on each occasion. The samples were then kept for 1 h in (50% Histolene plus 50% absolute ethanol). In the final step, the samples were cleared in Histolene and wax solution (twice) for 2 h each, respectively. The whole process took 16.5 h.The tissue samples were then embedded in wax using a Tissue-tek- 11 Tissue Embedding Center (model 4603) designed for the paraffin method of tissue embedding. This provided accurate sample orientation during the mold processing and a refrigerated plate for solidifying

paraffin samples. Samples in 8 µm thickness were prepared from these wax embedded samples using a Leitz Rotary Microtome (model 1512). These were then mounted on glass slides coated with poly-L-lysine. All slides were stored in the slide boxes to protect them from contamination.

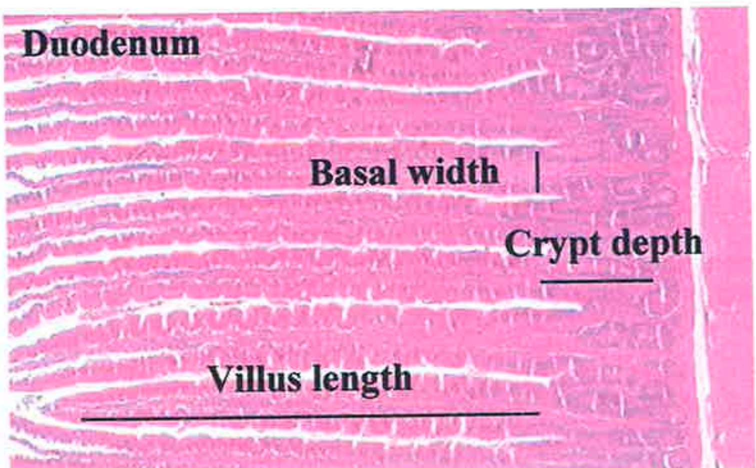
2.7. Intestinal morphometry

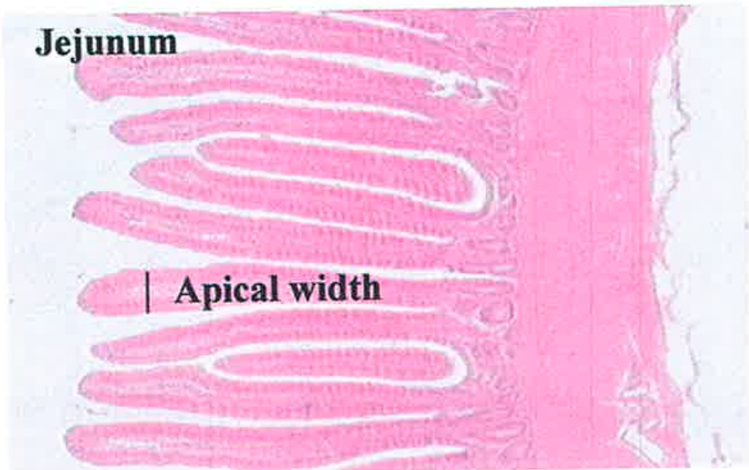
Intestinal samples (duodenum, jejunum, ileum) were processed for staining for morphometry studies as follows: Sections (tissue on glass slides) were placed in an oven at 60 C° for 40 min for dewaxing. They were then cleaned in histolene solution twice for 3 min each. In the next step, all slides were rehydrated through graded ethanol (absolute ethanol, 80% ethanol, 30% ethanol) for 2 min in each solution, then rinsed in distilled water for 20 seconds. The slides were stained in haematoxylin for 5 min then rinsed in tap water and 70% ethanol and 1% HCL acid at once and for 5 and 1 sec, respectively. They were then washed in flowing tap water for 10 min stained in Eosin for 1 min, then washed in tap water twice. They were then dehydrated in 80% and absolute ethanol once at 5 sec and twice at 2 min respectively. At the final stage, the slides were cleared for 5 min in Histolene. They were then mounted with depex, covered with coverslips, and morphometrically measured. Villus height and crypt depth (µm) were measured using an Image video program (Video pro 32 Color Image Analysis Leading Edge SA) and Olympus-BH-2 Microscope. The surface area of villi (mm^2) was calculated by following equation (Plate.2.1)

$$Villus\ surface\ area\ (mm^2) = \frac{(Apical\ villus\ length\ + Basal\ villus\ length,\ um)}{2x10^6} x\ Villus\ height\ (um)$$

The effect of estrogen, calcium, age and different diets on morphometry of the

Plate 2.1: Typical Haematoxylin and Eosin stained sections of duodenal, jejunal and ileal tissue taken from pre-lay hens. Morphometric measures were taken as indicated.


Duodenum
Basal width
Crypt depth
Villus length


Jejunum
Apical width

Ileum

intestinal regions (duodenum, jejunum and ileum) were determined by this procedure.

2.8. Immunohistochemical methods

For the study of calbindin D_{28k} and estrogen receptor localisation, all tissue samples were placed in 70% ethanol after 24 hours fixation in Carnoy's or formalin respectively.

Specific mouse monoclonal antibodies, against chicken calbindin D_{28k} (anti-calbindin-D, Clone Cl-300 Sigma) was used as the primary antibody against Calbindin D_{28k} and mouse monoclonal anti-human estrogen receptor (ER, 1D5, DAKO) was used as the primary antibody against ER of laying hens (Kumar *et al.*, 1987). Non- specific mouse IgG1 (Sigma, code No. X931) was used as a negative control. All antibodies were detected by biotinylated sheep anti-mouse IgG as a secondary antibody. This antibody was prepared by hyper-immunizing sheep with purified immunoglobulin from normal serum of mouse. This antibody is conjugated with horse radish peroxidase (HRP) which in the presence of hydrogen peroxide (H_2O_2) and diaminobenzidine tetrahydrochoride (DAB) producers a brown insoluble reaction product. The product precipitates at the side of reaction thereby allowing localisation of the product in the tissue.

2.8.1. Estrogen receptor localisation in jejunum tissue

The following procedure was used in the estrogen receptor localisation in jejunal tissue: All slides were placed in the oven for 30 min. at 60°C for dewaxing and incubated twice in histolene for 5 min on each occasion. They were then rehydrated through graded alcohols (absolute ethanol 80% ethanol, and 30% ethanol and

distilled water, 2 min each). Slides were finally incubated in 0.07% acid ethanol for 5 min to block endogenous (H_2O_2)

The slides were rinsed in phosphate buffer saline (P.B.S, pH 7.4) plus 2% gelatine for 30 min at room temperature for blocking non-specific antibody binding in tissue. The procedure was continued by incubating the slides in a citrate buffer (pH 6) at 92-98°C in a microwave for 10 min (this step was divided into two 5 min stages and the plastic jar which contained the slides refilled by citrate buffer between these two stages). The slides were then cooled at room temperature for 20 min. The temperature in this stage breaks bounds formed during fixation to expose the antigen reactions sites.

The primary antibody and negative control were diluted in 1/100 P.B.S. (pH 7.4) with 2% gelatine as described in this chapter. All samples were covered by coverslips and kept at 4°C overnight in a humidified chamber. The coverslips were removed very slowly with P.B.S. then slides rinsed in P.B.S buffer (pH 7.4.) Biotinylated anti-mouse IgG, as (previously described) was used as the secondary antibody and diluted with 1/100 P.B.S. The slides were then covered by coverslips and placed in a humidified glass chamber at room temperature for 2 h. The coverslips were then removed by P.B.S and the slides rinsed in P.B.S. Vectastain Elite ABC reagent was used for increasing sensitivity and reducing staining times. All tissues were covered by coverslips and kept for 1 h in a glass chamber at room temperature.

In the next step the slides were placed in P.B.S buffer after the coverslips were removed and incubated for two 5 min periods. The slides were put in

diaminobenzidine tetrahydrochoride (DAB 0.5 mg /ml P.B.S. pH 7.4) containing 0.04% H_2O_2 . They were kept from light for 2 min at room temperature, then washed in tap water and mounted with depex.

2.8.2. Calcium binding protein localisation in the jejunum tissue

A similar protocol to ER localization was used for calbindin D_{28k} determination. However, the primary antibody (anti-ER, 1D5, DAKO) was substituted with sheep anti mouse IgG agent D_{28k} and no antigen exposure was needed to be performed.

2.9. Jejunum homogenate and enzymes activities

Approximately 2-4 g tissue sections which had been frozen in liquid nitrogen were defrosted in 25 ml buffer (100 mM Mannitol, 2 mM Hepes, pH 7.1), cut into small pieces, vibromixed for (2x30 sec) then coarsely filtered through a buchner funnel. The filtrate was mixed in a in a waring blender for 30 sec. The final homogenate was stored in 2 ml screw capped tubes in liquid nitrogen for further study. Protein, alkaline phosphatase, Ca ATPase, Mg ATPase, Ca/Mg ATPase and Na/K ATPase levels in the homogenate were assessed in the samples.

2.9.1. Protein determination

Protein was determined by the method of Bradford (1976). Bovine Serum Albumen (BSA) was used as the standard.

2.9.2. Alkaline phosphatase assay

Homogenates of jejunum samples were stored in 2 ml screw capped tubes in liquid nitrogen were used for alkaline phosphatase (AP) assay. In both samples and standard

(25 µl) were diluted (1:20) in distilled water for jejunum enzyme activity based on the method of Forstner *et al.* (1968). The assay was carried out as follows:

In samples preparation, 800µl of 50 mM Tris buffer (pH, 10.1), 100 µl of 50 mM $MgCl_2$, 25 µl of diluted sample and 100 µl of 10 mM Sigma 104 phosphatase substrate were placed in disposable culture tubes (12 x 75mm). Blanks comprised 800 µl of 50 mM Tris buffer (pH, 10.1), 100 µl of 50 mM $MgCl_2$ and 125 µl distilled water in the similar tubes.

Incubated standards consisting of 100 µl of Sigma p-nitrophenol (10 µmole/ml concentration) and 25 µl distilled water plus 800 µl of 50 mM Tris buffer (pH, 10.1) and 100 µl of 50 mM $MgCl_2$.

The samples, blanks and standards were allowed to stand for 20 min at room temperature after which the reaction was stopped with 100 µl of 40% trichloroacetic acid (TCA). Then 100 µl of samples, blanks and standards were pipetted into fresh tubes and 2 ml of 0.4 n NaOH added. The mixture was vibromixed and read at 410 nm in Shimadzu Spectrophotometer UV-120, JAPAN.

2.9.3. Ca, Mg, Ca-Mg ATPase assay

All ATPase assays employed were based on the methods of Quiley and Gotterer, (1969); Qin and Klandrof (1993) and Qin *et al.*, (1993), Ca, Mg and Ca/Mg ATPase activity were determined in jejunum samples.

To determine the Ca/Mg ATPase activity, 40 µg of homogenate was added to 400 µl

of ATPase buffer (100 µl of 100 mM Tris-HCl, 100 µl of 25 mM $CaCL_2$, 100 µl of 50 mM $MgCl_2$ and 100 µl of 2.5 mM EGTA, pH 7.4) and diluted to 900 µl with distilled water. The ATPase assay mixture was incubated for 5 min at 40° C. The assay was initiated by the addition of 100µl ATP (30 mM adenosine triphosphate, disodium) and mixtures were incubated for 30 min at 40°C. The reaction was terminated by 100µl 40% TCA.

The ATPase assay mixture was vortexed and centrifuged at 2500 g for 5 min at 4° C and 0.25 ml of the supernatant was removed and placed in disposable culture tubes (12 x 75 mm). Distilled water (1 ml), 0.063 ml Fiske and Subbarow solution ((($(NH_4)_6$ $MO_7)_{24}$. $4H_2O$ in1 M H_2SO_4)) and 0.25 ml acid molybdate were added to the supernatant. Finally the mixture was incubated at room temperature for 20 min. The absorbance was read in the same Spectrophotometer at 660 nm.

The samples were measured against a standard which consisted of 0.25 ml of phosphorous standard solution (20µg/ml concentration). This was added to 1 ml of distilled H_2O, 0.063 ml Fiske and Subbarow solution and 0.25 ml of acid molybdate in culture tubes. It was read at 660 nm. Distilled water (1.25 ml) and the same volume of Fiske and Subbarow and acid molybdate were used as blanks and read at 660 nm. The incubation mixture (without tissue) was used as blanks for samples.

Mg-ATPase activity was assessed as described for Ca/Mg-ATPase except that $CaCl_2$ was omitted from the ATPase buffer assay. A similar assay was carried out for Ca-ATPase activity with 5 mM $CaCl_2$ in the absence of $MgCl_2$.

2.9.4. Na/K ATPase assay

A sample of jejunal homogenate (25µl) was prepared as follows:

Solution 1 (30 mM of Tris pH 7.1, 7.5 mM of $MgCl_2$); solution 2 (100 ml of solution 1, 120 mM of NaCl and 20 mM of KCl); solution 3 (50 ml of solution 2, 1 mM of ouabain (as inhibitor of Na/K ATPase activity). The incubation medium consisted of 25 µl of sample and 875 µl of one of the solution 1, 2, 3 in three separate culture tubes. Blanks consisted of 900 µl of each three solutions dispensed into culture tubes without sample materials.

All solutions were pre-incubated at 40°C for 5 min in a (Thermonox water bath model 1441) then the reaction started by adding 100 µl ATP (50 mM ATP). They were then incubated at 40 °C for 30 min. The reaction was stopped with 100 µl of 40% TCA. The rest of this assay was the same as that for Ca/Mg ATPase assay. Finally all assays were read at 660 nm. The ouabain (1mM) was used as Na/K ATPase inhibitor with solution 3. Therefore, the activity of Na/K ATPase was obtained by taken the reading results of solution 3 from solution 2, which described in this chapter. The data were subjected to ANOVA (analysis of variance) and mean values compared by LSD.

CHAPTER THREE

SMALL INTESTINAL EXPRESSION OF CALCIUM BINDING PROTEIN (CALBINDIN D_{28K}) AND ESTROGEN RECEPTORS IN 15 AND 26 WEEK OLD ISA-BROWN HENS

"Estrogen may have a physiological role in regulating intestinal calcium absorption" *(Arjmandi et al., 1993)*

3.1. Introduction

Calcium binding protein (CaBP, particularly calbindin D_{28k}) and estrogen receptors (ER) have been recognised to play a major role in many life processes including development, modulation of reproduction and numerous other physiological processes (Jande *et al.*, 1981; Korach *et al.*, 1996) Both (calbindin D_{28k} and ER) have been detected in oviduct and reproductive systems, but there is doubt as to the expression of ER in the intestine of birds, particularly in laying hens (Kusuhara and Ohashi, 1991; Wu, *et el.*, 1994). ER has been found in the intestinal mucosa of postmenopausal women (Arjmandi *et al.*, 1993).

It has been thought that these two factors (calbindin D_{28k} and ER) may effect calcium absorption and in particular during the onset of lay in laying hens. It is well established that there is an increase in calcium requirement during the sexual maturation and egg production in laying hens (Gilbert, 1983, Wu *et al.*, 1994). This increase is reflected in altered enterocyte calbindin D_{28k} expression, essential for calcium transport and increased capacity of the intestinal mucosa to absorb this nutrient in 25 week old hens compared with 11-17 week old hens (Wu, *et el.*, 1993, 1994). However, whether there is a direct action of estrogen on intestinal mucosa during sexual maturation is still in debate. The direct action of this hormone on intestinal tissue may be related to the expression of estrogen receptors in this region. Therefore, this study is aimed at identifying in the temporal and spatial expression of calbindin D_{28k} and estrogen receptors to determine the possibility of a direct action of estrogen on the jejunal of the small intestine.

This chapter focuses on the detection of CaBP (calbindin D_{28k}) and ER in the jejunum

of the small intestine of Isa-brown hens aged 15 and 26 weeks to show general physiological reaction of birds in this particular case. Intestinal mucosa structure, alkaline phosphatase and ATPase activity are also assessed in this chapter.

3.2. Materials and Methods

Ten Isa- brown hens, 15 and 26 weeks of age (5 birds of each group) were collected from a commercial flock. Birds were euthanased and their small intestine rapidly removed using the same procedure as previously described in chapter 2. CaBP (calbindin D_{28k}) and ER expression were detected by specific antibodies, intestinal mucosa morphometry and jejunal homogenate enzymes activities were measured as elucidated in Chapter 2.

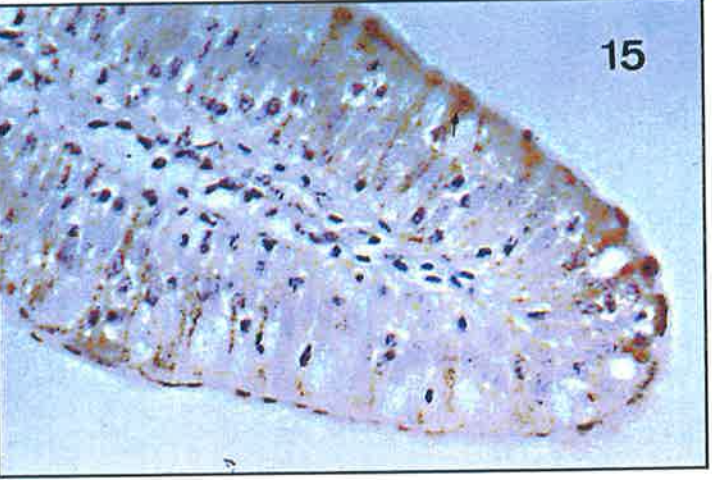
3.3. Results

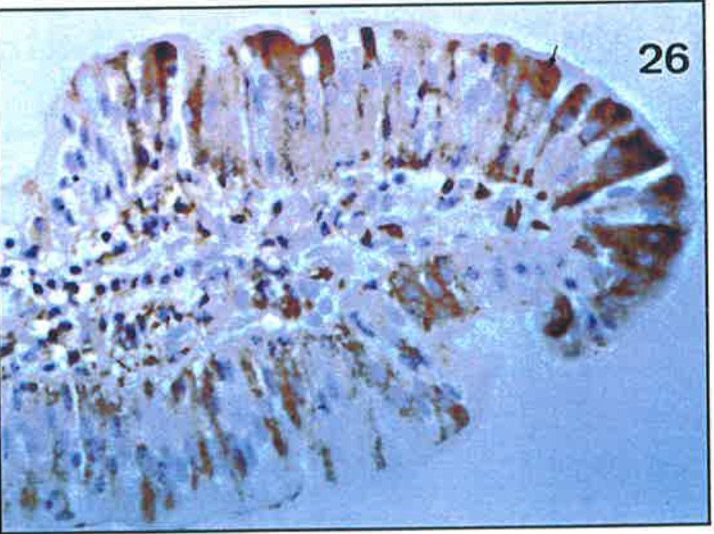
3.3.1. Localisation of calcium binding protein (calbindin D_{28k}) and estrogen receptor

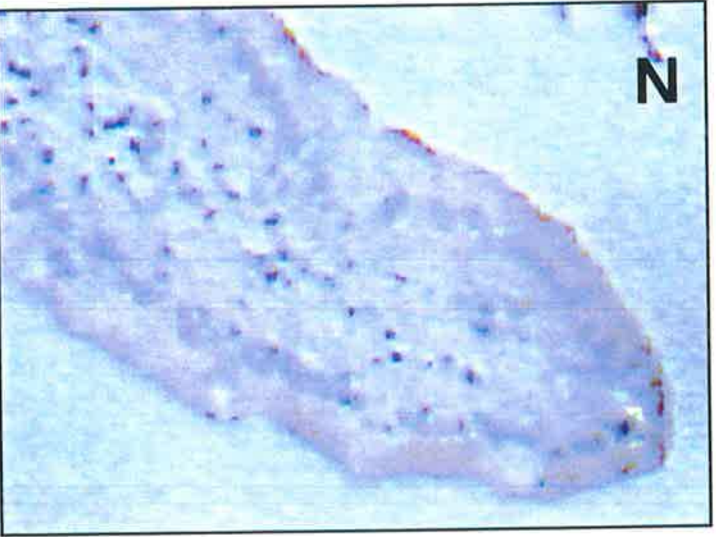
Expression of calbindin D_{28K} in both 15 and 26 week old hens in jejunal tissue are shown in plate 3.1. Jejunum tissue as a being the largest site in small intestine has been tested to observe the expression of calbindin D_{28K} with respect to age. A greater expression of calbindin D_{28K} was detected in cytoplasm and nucleus in absorptive cells of enterocytes a long the length of villi in 26 weeks birds compared with 15 weeks old birds. In contrast, regardless of age, immune staining for estrogen receptors was observed to be predominantly in cytoplasm in villus tip entrocytes* jejunal tissue. A similar distribution pattern was observed in regard to expression of ER in jejunal tissue of 15 (before onset of lay hens) and 26 weeks old hens (Laying hens).

Plate 3.1: Immunohistochemical localisation of estrogen receptors (A) and Calbindin D28K (B) expression in typical jejunum sections from 15 and 26 week old Isa Brown hens. Primary antibodies were detected using a Streptavidin - Horseradish peroxidase system (section 2.8 chapter 2). Arrows indicate positive reactions in epithelial cells. N indicates sections incubated without the primary antibody (negative controls).

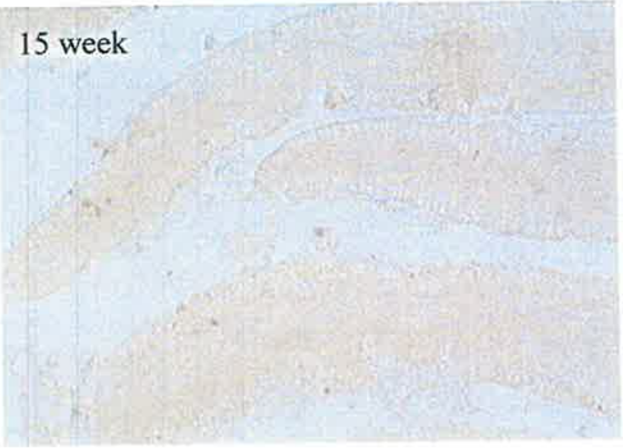
A

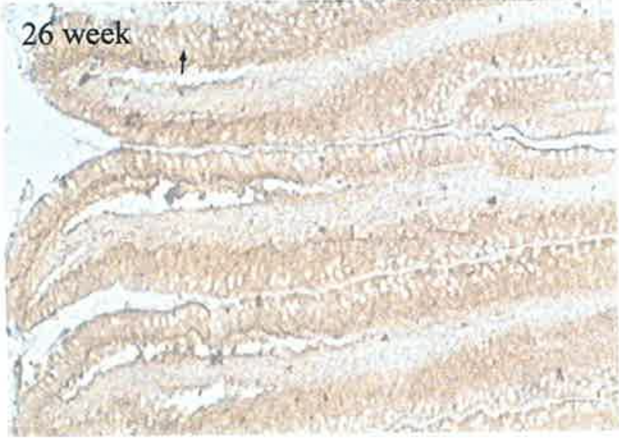


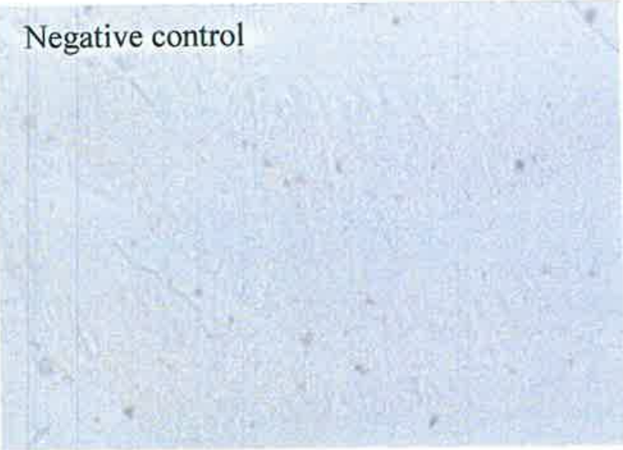




B







3.3.2. Intestinal mucosa morphometry

Data for intestinal mucosa structure of both groups of birds are shown in Table 3.1. Although duodenum surface area in 26 week old hens was greater ($P<0.05$) than 15 weeks old, no significant differences were observed in 15 and 26 weeks Isa-Brown hens with crypt depth, villus height and villus surface area in different regions of the small intestine. Irrespective of the age the villus characters were reduced from the duodenum to ileum (Table 3.1)

Table 3.1: Intestinal mucosa structure in 15 and 26 week old Isa- brown hens

	weeks age		
	15	26	SEM
A. Crypt depth (µm)			
Duodenum	330.10	334.20	8.76
Jejunum	269.00	296.00	26.20
Ileum	220.00	210.00	22.50
B. Villus height (µm)			
Duodenum	1438.00	1662.00	106.00
Jejunum	1212.00	1242.00	48.00
Ileum	914.00	988.00	37.00
C. Villus surface area (mm^2)			
Duodenum	271.00^b	370.00^a	31.30*
Jejunum	168.10	161.90	13.77
Ileum	128.20	123.80	4.46

Mean values on the same row with different superscript are significantly different*, ($P<0.05$); SEM, standard error of means.

3.3.3. Enzyme activity in jejunal homogenate

Alkaline phosphatase and Ca, Mg, Ca/Mg and Na/K ATPase activities of homogenate of jejunal tissue of the small intestine are shown in Fig. 3.1.a and 3.1.b respectively. Expression of alkaline phosphatase was significantly greater ($P<0.05$) in 26 week old than in 15 week old hens (Fig. 3.1 a). No significant difference was shown in ATPase activity between 15 and 26 week's age. Although it appears Na/K ATPase activity was reduced remarkably (approximately 50%) with age (Figure 3.2).

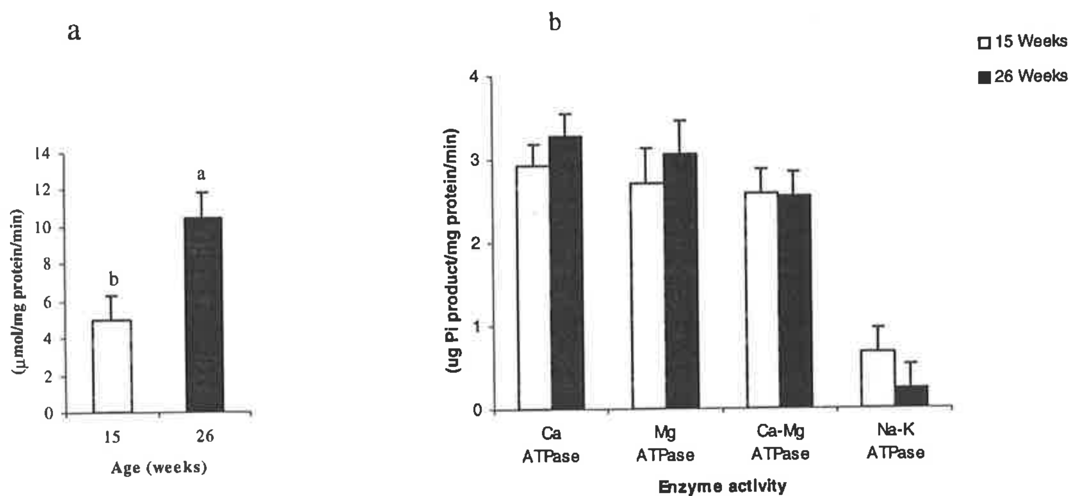


Figure 3.1: (a) Alkaline phosphatase activity in jejunal homogenate at 15 and 26 week old Isa-brown hens; (b), ATPase enzyme activity in jejunal homogenate at 15 and 26 week old Isa-brown hens; Columns with different letters within the enzyme activity by age (15 and 26 week old) differ significantly for alkaline phosphatase ($P<0.05$).

3.4. Discussion

In this study expression of CaBP (calbindin D_{28k}) and ER were determined in jejunum tissue of different age (15 and 26 weeks) Isa-brown hens. Expression of CaBP (calbindin D_{28k}) in the intestine and particular jejunum tissue is in agreement with the results of others (Jande *et al.*, 1981; Bar *et al.*, 1990; Wu *et al.*, 1994). Estrogen receptor localisation in the jejunum tissue irrespective of age was detected for the first time in the cytoplasm of hens, although this has been identified in human intestine (Arjmandi *et al.*, 1993). The expression of estrogen receptor in the nuclear of oviduct in immature hens has been localized by Kusuhara and Ohashi (1991). The demonstrated existence of estrogen receptor in the intestine, particularly in the jejunum tissue, shows that there is a potential to affect calcium absorption at this site. Expression of ER in small intestine regardless of age shown that ER expression precedes calbindin D_{28k} thereby demonstrating that estrogen has the potential to regulate calbindin D_{28k} expression in this tissue. Therefore increased estrogen level during sexual maturation may induce calbindin D_{28k} expression and hence modulate

intestinal absorptive capacity for calcium. Findings of Krust, *et al.* (1986) indicate that steroid hormone receptors bind to specific DNA elements and activate transcription and also Piccotto *et al.* (1996) have reported that there is a possible non-genomic pathway of E_2 for regulating calcium transport in rat. This may modulate calbindin D_{28k} and ATPase activity as main factors for calcium transport in plasma membranes and inside the cell.

Alkaline phosphatase (AP) activity in jejunum increases with age in Isa-brown hens as does surface area of the duodenal villus. This increase in AP may be related to a greater calcium absorption at 26 weeks old hens during egg production and shell formation, compared with 15 weeks old hens as a non-egg producing group as described by Nys and Laage (1984). In addition, significant increases in villus surface area and villus height in different regions of small intestine may correspond with increase in the cell size of villus entrocytes and this might result in a greater capacity for protein synthesis, particular proteins which are essential for calcium absorption (calbindin D_{28k}). The expression of calbindin D_{28k} in this study was localised in the cytoplasm of absorptive cells such distribution has been observed in other studies (Jande *et al.*, 1981; Spencer *et al.*, 1978; Wu *et al.*, 1993).

In conclusion the expression of ER precedes calbindin D_{28k} in jejunal intestine tissue indicating that estrogen has the potential to influence the expression of calbindin D_{28k} in small intestine during the onset of lay and this may increase calcium absorption in this particular case.

CHAPTER FOUR

INTESTINAL FUNCTION AND REPRODUCTIVE CAPACITY OF 18-22 WEEK OLD TEGEL PULLETS IN RESPONSE TO ADMINISTRATION OF EXOGENOUS ESTROGEN

"Increased calcium transport in laying hens could involve interaction between estrogen and estrogen receptors" *Wu et al. (1994).*

4.1. Introduction

The efficiency of any feeding program is dependent on it meeting all the nutrient requirements of the bird. Therefore, before feed formulation is undertaken, specific nutrient requirements must be determined. One of the essential and important nutrients in laying hens is calcium.

The amount of feed consumed by poultry is dependent on the energy level of the feed, particle size of the feed components, the body weight, rate of egg production of the hens and environmental temperature (Austic and Nesheim, 1990; Hunton, 1995). All of these factors can affect calcium consumption in laying hens. In general, poultry eat to satisfy their energy needs (Scott *et al.*, 1982; Hunton, 1995). Reducing the fibre content or increasing fat alters the energy level of feed. If the energy level of feed is increased, a corresponding increase in protein, vitamins and minerals is necessary for a balanced ration, since birds consume less of high-energy feed. This situation, however, may change the amount of calcium consumed in laying hens.

It has been thought by Wu *et al.* (1994) that the interaction between estrogen receptors in the intestine and exogenous estrogen may have a effect on calcium transport and consequently may influence calcium retention, egg and eggshell quality. Based on this hypothesis, daily administration of 17β-estradiol (E_2), a synthetic estrogen, by injection with peanut oil as a carrier, may affect energy requirement and consequently might influence feed intake and feed efficiency.

Changes in feed efficiency may effect the retention of a major nutrient such as calcium which can affect egg production, particularly eggshell quality, as the calcium requirement increases after the onset of lay. It is widely known that synthesis of 1,25-dihydroxyvitamin D_3(1,25$(OH)_2D_3$) which is essential for calcium absorption may be increased by indirect effects of estrogen on the kidney at onset of lay hens (Wu *et al.*, 1994). In addition, estrogen level has long been recognised to increase during sexual maturity in pullets (Hurwitz, 1989). The development of secondary sexual characteristics and gonads is also accomplished by endogenous estrogen secretion (Lien and Cain, 1987).

There is however, conflicting results regarding the effect of exogenous estrogen on egg production and eggshell quality in aged Leghorn hens. Certain studies (Grunder *et al.*, 1980 and 1981) have shown an increase in eggshell thickness, egg weight and oviduct weight under the influence of exogenous estrogen, on 32-week-old hens. In contrast no response has been found to exogenous estrogen administration in the same strain (Leghorn) and similar age (32 weeks), (Li *et al.*, 1986; Qin and Kandorf, 1995). The majority of these studies have been conducted on aged Leghorn laying hens. However, limited research has been performed on young birds (pre-and point-of-lay of hens). There are no reports regarding the effect of estrogen in Tegel pullets. In addition, Tegel is one of the common strain which is using in the most poultry farms in Australia.

The variability of response to exogenous E_2 may be due to the administration of E_2 at pharmacological rather than physiological levels (Sommerville *et al.*, 1989). Estrogen treatment may also be ineffective if the administration period is short term (Li *et al.*, 1986). Furthermore, administration of estrogen, either in the diet or by

implant has been shown to be ineffective due to the dilution effect with other nutrients and tissues (Qin and Kandorf, 1995). Based on these reviews, the current study was intended to examine long term injection of estrogen on intestinal function (mucosa structure, feed efficiency, enzymes activity and calbindin D_{28k} expression) and the capacity of reproduction system (oviduct and shell glands potential, egg and eggshell characteristics) and also the relationship of these parameters. Therefore, this chapter will cover the effect of estrogen on calcium retention as critical nutrient in laying hens and its relationship to egg, eggshell characteristics, enzyme activities and the expression of calcium binding protein during the onset of lay in 18-22 week old Tegel pullets.

4.2. Materials and methods

Thirty-six Tegel pullets (18 weeks) of Red Island x Australope were kept in individual cages for four weeks. Rearing condition, temperature, humidity, drinking water and lighting (14 hours light and 10 hours dark) were identical for all treatments. Three treatments were used in a complete random design (3 x12). Intramuscular injections of estrogen levels at 0, 10 and 100 μg/kg body weight carried by peanut oil and were administrated daily at 5 pm. The constant calcium level in diet (3.72 %) with different levels of estrogen (0, 10, 100 μg) was considered for study of nutrient retention. including; dry matter (DM), crude protein (CP), Ca, P, Na, K and Mg. Daily feed intake (DFI), weight gain (WG), oviduct weight (OW) and feed conversion ratio (FCR) regarding egg production. The first egg was laid at the mid of the second week of treatment. The eggs were collected every afternoon at 4 pm for 20 successive days. Egg and eggshell characteristics, alkaline phosphatase and ATPases activities and expression of calbindin D_{28k} were measured based on methods described in chapter 2. The composition of feed provided for the hens based

on National Research Council (NRC, 1994) recommendations was:

Feed formulation for 18-22 week old Tegel pullets (diet composition g/100g)

Feed sources	
1. Wheat	70.00
2. Barley	10.00
3. Meat meal (50% CP)	11.70
4. Lysine (synthetic)	0.25
5. Methionine (synthetic)	0.12
6. Marble (Calcium source)	7.20
7. Sunflower oil	0.38
8. Sodium Bicarbonate	0.02
9. Mineral Vitamins & Yolk colorant	0.33
Total	**100.00**
Metabolism energy (ME)[1]	11.44 MJ/kg
Crude protein[2]	17 %
Calcium[2]	3.72 %
Total phosphorous[2]	0.73 %
Fibre[2]	2.52 %
Fat[2]	3.65 %

1. calculated, 2. measured; as described in chapter 2.

The data were subjected to ANOVA and mean values compared with LSD.

4.3. Results

4.3.1. Feed efficiency and growth

Birds had an initial weights (IBW) of 1.56-1.62 kg and exhibited weight gains (WG) of 308-425 g over the four weeks of treatment. No significant effect of treatment was observed but increasing the concentration of E_2 injected tended to reduce weight gain and oviduct weight per 100 g of body weight (Table 4.1). Such trends are reflected in daily feed intake (DFI) and feed intake per 100 g body weight (FI) although again the differences observed in these cases that were not significant. Feed conversion ratio (FCR) was improved in 10 μg of E_2 compared to 0 and 100 μg kg body weight/day of E_2, however there was still no significant differences between treatments (Table: 4.1).

Table 4.1: The effect of estrogen on growth and feed intake in 18-22 week old Tegel pullets

	Estrogen (μg/kg body weight/day)			
	0	**10**	**100**	**SEM**
A. Growth				NS
Initial body weight (IBW, kg)	1.56	1.62	1.58	0.09
Weight gain (WG, g)	425.00	393.00	308.00	52.40
Oviduct weight (OW, g/100g body weight)	2.07	1.81	1.75	0.34
B. **Feed intake**				NS
Daily feed intake (DFI, g)	122.00	111.70	104.50	8.96
Feed intake (FI, g/100g body weight)	169.40	165.6	159.6	7.35
Feed conversion ratio (FCR, egg, g/ feed, g)	2.94	2.73	2.77	0.34

SEM, Standard error of mean; NS, not significant.

4.3.2. Nutrients retention

The effect of estrogen on the retention of DM, CP, Ca, P, Na, K, and Mg is shown in Fig 4.1. Significant effects ($P<0.05$) were measured at a dose of 10 μg/kg body weight /day of E_2 on crude protein and potassium retention compared with both 0 and 100 μg of E_2. Estrogen doses also reduced Ca retention, in this trend, 100 μg of E_2 significantly ($P<0.05$) reduced Ca retention compared with 10 or zero dose (Fig. 4.1). Although it appeared that the retention of Ca was not significantly different between 0 and 10 μg of E_2. No significant effect of estrogen levels (0,10 and 100) were shown in dry matter, phosphorous, sodium, and magnesium retentions.

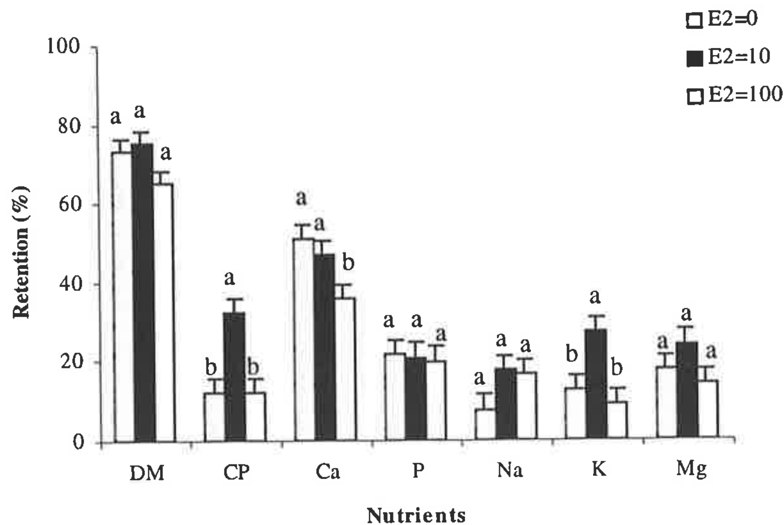


Figure 4.1 Nutrient retentions in response to different levels of estrogen. Data are means ± SE of 36 Tegel pullet at 0, 10 and 100 μg/kg body weight/day of Estrogen (E_2). Columns with different letters within nutrient differ significantly (P<0.05).

4.3.3. Egg and egg shell characteristics

The effect of E_2 on egg and eggshell characteristics are shown in the Table 4.2. Shell thickness and weight of shell per unit of surface were significantly greater (P<0.05) with treatment 10 μg of E_2 compared with either 0 or 100 μg of E_2 after 4 four weeks of treatment. There were no significant differences between the groups for egg production (EP), egg weight (EW), egg mass (EM) and specific gravity of egg (ESG) at different levels of estrogen administration, although egg production with both 10 and 100 μg of E_2 (27.60 and 26.60% respectively) are greater than 0 (20%) treatment. Shell weight (SW), shell weight as percentage of egg weight (SP) and surface area of egg shell (SFA) showed no significant response to estrogen level in this stage of development (22 weeks Tegel pullets).

Table 4.2: Egg and egg shell characteristics response to estrogen levels in 18-22 week old Tegel pullets.

	Estrogen (µg/kg body weight/day)			
	0	10	100	SEM
A. Egg characteristics				
Production (EP, egg/hen/house)	20.60	27.60	26.50	6.49 ^{NS}
Weight (EW, g)	43.60	41.20	39.50	1.86 ^{NS}
Mass (EM, g/day)	12.30	16.20	12.10	2.62 ^{NS}
Specific gravity (ESG)	1.0913	1.0973	1.0957	0.0015 ^{NS}
B. Egg shell characteristics				
Weight (SW, g)	4.63	4.79	4.25	1.59 ^{NS}
Thickness (ST, µm)	354.20^{b}	393.50^{a}	356.6^{b}	3.14 *
Surface area (SFA, cm^2)	56.27	54.32	52.82	1.57^{NS}
Percentage (SP, %)	10.96	11.58	10.69	0.17^{NS}
Weight/unit area (WU, mg/ cm^2)	82.48^{b}	88.22^{a}	80.66^{b}	0.70 *

Mean values on the same row without a common superscript are significantly different, *, ($P<0.05$); SEM, standard error of means; NS, not significant.

4.3.4. Intestinal mucosa morphometry

Intestinal mucosa structure has been tested for that level of estrogen (10 µg) which showed more response on nutrient retention and shell thickness compared with control group (0 µg of E_2). Within any given region, villus height, crypt depth and surface areas were not affected by 10 µg/kg body weight/day of estrogen when compared with control hens (Table 4.3). Regardless of treatment villus height, crypt depth and surface area declined distally from the duodenum to the ileum.

Table 4.3: Intestinal mucosa structure in 22 week old Tegel pullets in response to estrogen levels

Segments	Estrogen (μg /kg body weight/day) 0	10	SEM
A. Crypt depth (μm)			NS
Duodenum	395.00	372.00	19.50
Jejunum	361.00	336.00	16.7
Ileum	272.80	275.70	12.38
B. Villus height (μm)			NS
Duodenum	2363.00	2374.00	111.70
Jejunum	2328.00	2052.00	155.30
Ileum	1435.00	1389.00	66.20
C. Villus surface area (mm^2)			NS
Duodenum	0.764	0.733	0.066
Jejunum	0.615	0.520	0.062
Ileum	0.343	0.319	0.011

SEM, standard error of means; NS, not significant.

4.3.5. Enzymes activities of jejunal homogenate

Since there was a greater effect of 10 μg estrogen/kg body weight/day on nutrient retention and shell thickness, this level of estrogen was used to determine treatment effects on intestinal enzyme activities. No significant response to estrogen was detected in alkaline phosphatase (Fig. 4.2 a) and Ca, Mg, Ca/Mg and Na/K ATPases (Fig. 4.2 b), although the mean values of each values except Na/K ATPase, fell with treatment.

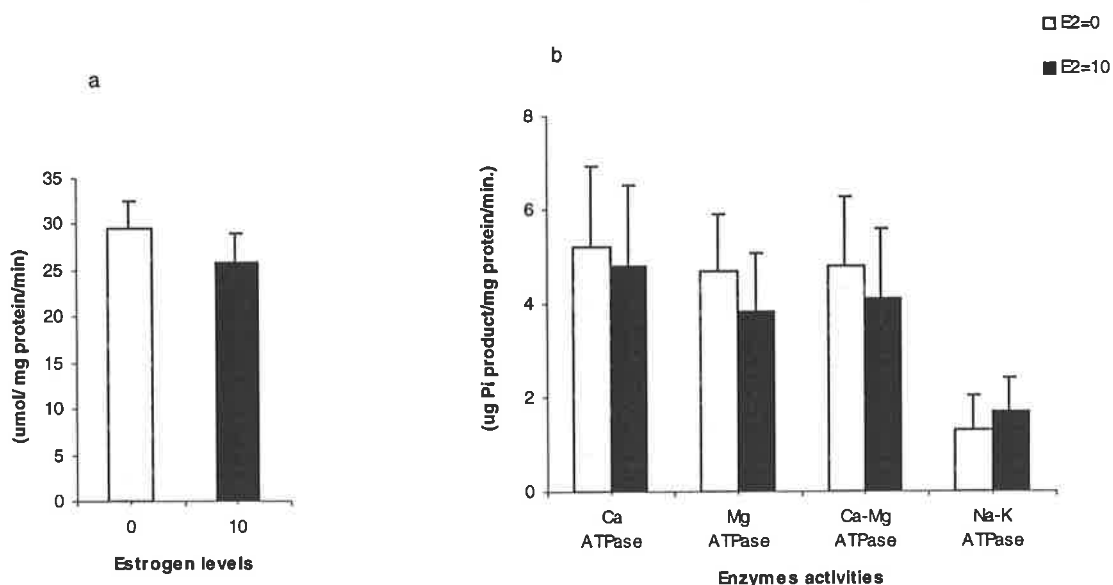
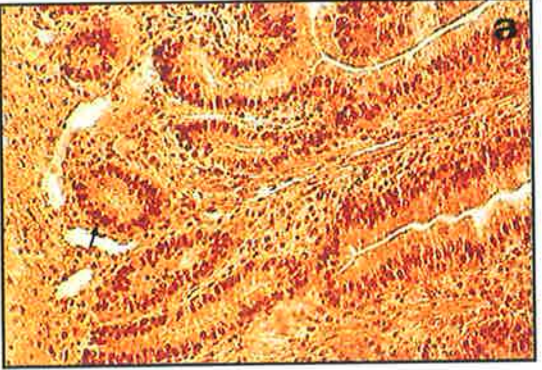


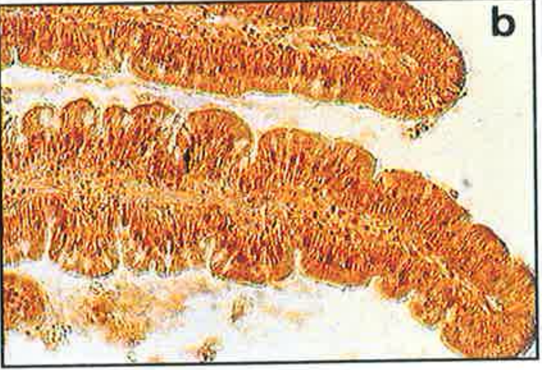
Figure 4.2: (a) Alkaline phosphatase activity of jejunal homogenate in 22 week old Tegel pullets; (b) ATPase activities of jejunal homogenate in 22 week old Tegel pullets. At 0 and 10 estrogen (E_2) µg/kg/body weight/ day. Data are means ± SE of 36 pullets.

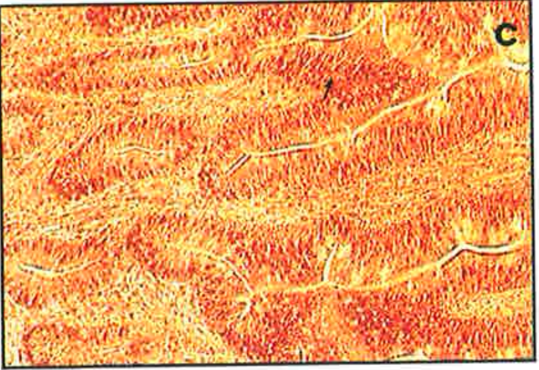
4.3.6. Calcium binding protein (calbindin D_{28k}) expression

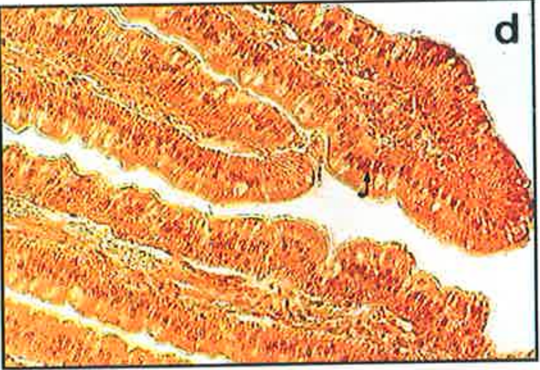
Localisation of CaBP (calbindin D_{28k}) expression in jejunal tissues was tested at the 0 and 10 µg E_2 groups (as described in this chapter) and are illustrated in plate 4.1. No difference was found in expression of CaBP (calbindin D_{28k}) at both levels of estrogen in the 22 week old of Tegel pullets. In general calbindin D_{28k} was localised in the both cytoplasm and nucleus of epithelial and absorptive cells in the enterocytes of small intestine at 22 weeks Tegel pullets. There was no detection of CaBP in the negative control groups.

Plate 4.1: Immunohistochemical localisation of Calbindin D_{28K} expression in typical sections from 22 week old Tegel pullets treated with exogenous estrogen. Panels a and b, respectively represent crypt and villus regions of jejunal tissue taken from control (0μg E_2/kg body weight/day). Effect of exogenous estrogen (50μg E_2/kg body weight/day) is represented in panels c and d. Negative controls (n) underwent the same treatment as test slides except the primary antibody was omitted (section 2.8. chapter 2). Arrows indicate typical positive staining in epithelial cells.


a


b


c


d

n

n

4.3.7. Discussion

This study determined a significant effect of long term administration 10 µg /kg body weight/day of E_2 on egg shell thickness and shell weight per unit of surface area, similar to results observed by Gunder *et al.*, (1981). Contrasting results reported by others Li, *et al.* (1986) and Qin and Klandraf, (1995), may be due to implants or the administration at too low a dose over a short duration. It seems that 10 µg/kg body weight/day of estrogen may improve shell thickness by 10% and weight of shell per unit surface area by 6.5%. Although the retention of protein and potassium were significantly greater ($P<0.05$) in 10 µg/kg body weight/day of estrogen group compared with 0 and 100 µg E_2, no differences were recorded with other nutrient retention. These points have not been reported in other studies with Tegel pullets.

No response was found with respect to estrogen effect on daily feed intake, weight gain, dry matter retention, FCR, and oviduct weight in this study, although increasing estrogen doses was coupled with a reduction in growth. Such findings have been shown by the others (Adams, *et al.*, 1950; Qin and Klandorf, 1993). The high E_2 dose (100 E_2) in this case (Tegel strain) could adversely affect the estrogen receptors. Consequently prevent stimulation of DNA for synthesis particularly the protein (calbindin D_{28k}) which is essential for calcium transport (Lien and Cain, 1987; Bar *et al.*, 1990). Non-genomic effect of estrogen receptor can not be ignored in this issue (Picotto *et al.*, 1996). Expression of CaBP (calbindin D_{28k}) in enterocytes of the jejunum tissue was not changed by any levels of estrogen. In contrast reduction of calcium retention in high dose of estrogen could be dependent on reduction of protein retention. These reductions were corresponded with a reduced the DM retention. Since the reduction in DM retention could lead to reduce of protein and consequently

in calcium retention.

Alkaline phosphatase and ATPase (Ca, Mg, Ca/Mg and Na/K) enzymes activities in jejunal tissue showed the same trend in all treatments, except in Na/K ATPase has shown a higher response in 10 µg /kg body weight /day of E_2 than the control group (0 µg of E_2).

No significant differences in retention of dry matter which was found between treatments in this investigation, may be related to the mucosal structure of intestine (villus height, surface area and crypt depth) which were not changed by 0, 10 and 100 µg of E_2 levels administration (Table 4.3). Since increase or decease of different parameters of villus in the intestinal structure may change the cell sizes of villus entrocytes thus potential affecting protein synthesis. This might change the DM or nutrients transport and consequently nutrient retention.

There is some evidence that interaction between endogenous estrogen and 1,25$(OH)_2D_3$ may induce calcium transport across the small intestine in aging hens (Wu *et al.,* 1994), thereby increasing the capacity of the animal to supply calcium to the body for maintenance and egg shell production. The observed effect of exogenous E_2 in the present study may involve such mechanisms. However, E_2 had a little effect on villus morphometry. Although significant effects ($P<0.05$) of 10 µg/kg body weight/day of estrogen was shown on egg shell thickness, the increased calcium absorption must have been due to a cellular mechanism rather than a gross structural change. Davis *et al.* (1979) have noted that mitochondria, golgi, endoplasmic reticulum and endosomal-lysosomes pathway play a vital role as carrier

for intracellular calcium transport. In addition in the chicken intestine microtubules, cytoskeletal elements along which vesicles move, have been shown to contain 1, 25$(OH)_2D_3$ regulated protein including calbindin D_{28k} (Rubinoff and Nellans, 1985). Therefore, an increased calcium absorption may be achieved by upregulating the rate of intracellular calcium movement, rather than an elevated number of carriers. In contrast, to increases egg shell thickness and shell weight per unit area at 10 μg of estrogen /kg body weight/day in 22 weeks old Tegel pullets, there was less response in calcium retention and lower activities in ATPase enzymes, particularly in Ca ATPase. However increased activity appeared in Na/K ATPase at this level of estrogen. Increasing eggshell thickness and shell weight may be due to ultrastructure of shell and effect of Na/K ATPase on calcium transport in cell membranes respectively. In addition, increase retention of protein in this case, may enhance calcium absorption, because high level of protein retention is associated with an increased of calcium absorption (Civitelli *et al.*, 1992).

It is clear that retention of protein increased significantly at the 10 μg level but not at 100 μg E_2. In contrast, reduced weight gain at this level of estrogen could be related to small differences in initial body weight, although differences in initial weights were not significantly identified. However the slight differences in initial body weight could not have influenced feed intake significantly.

In conclusion, administration of estrogen at 10 μg /kg body weight/day increased shell thickness (10%) and weight per unit area (6.5%). Calcium retention, weight gain, and ATPase activities were reduced by this particular estrogen dose (10 μg). Increases in shell thickness and shell weight per unit area, may be due to architecture

of the ultrastructure of shell and an increase in calcium absorption with respect a high retention of protein at 10 μg of E_2.

The question arises, what are the effects of the other levels of estrogen, different amounts of calcium in diets and finally the influence of different strains. The following experiment considers these three aspects.

CHAPTER FIVE

COMPARISON BETWEEN 18-22 WEEK OLD LEGHORN AND TEGEL PULLETS FOR INTESTINAL FUNCTION AND REPRODUCTIVE CAPACITY IN RESPONSE TO EXOGENOUS ESTROGEN AND DIETARY CALCIUM.

"Egg shell quality is of major importance to the Australian poultry industry."

(Roberts and Brackpool, 1995)

5.1. Introduction

Egg quality is highly dependent on eggshell quality. The properly structured shell is an efficient barrier against the transmission of micro-organisms and fracture, thus making the egg a satisfactory place for the development and growth of embryo (Solomon, 1991; Standelman, and Cotterill, 1995).

Eggs need to be protected from pathogens and damage during collections, packaging and transportation. This is achieved by good shell formation, which is in turn, dependent upon the calcium carbonate content in the shell. Therefore calcium is one of the key elements essential for maintenance and reproduction of laying hens. Calcium is the most abundant inorganic component of the skeleton and plays a major role in a wide variety of biological functions. Commercial laying hens lay about 280 eggs during the laying cycle, each weighing approximately 60g. This high rate of lay can lead to a depletion of calcium in the eggshell. Eggshells that weigh 4-6 g contain about 2 g calcium. This amount of calcium (in one egg) is equal to 10% of the total calcium content of the pullet's body (Gilbert, 1983). Therefore during one year of egg production, calcium loss is equal to about 30 times the hens total body calcium (Gilbert, 1983). This high demand of calcium is not only dependent on a high rate of calcium metabolism, but also on the genetic potential of pre-laying hens. Although the heritability of shell thickness in laying hens has been estimated to be between 25-60% (Dalton, 1985), the contribution of poultry strain in this variation has not been elucidated. The effect of this variation in heritability on shell quality and consequently calcium retention are not known. The interaction effects of estrogen and dietary calcium, on calcium retention and consequently on egg and shell quality in hens of different strain, still remains unclear. This chapter examines the effects of different levels of estrogen and various percentage of dietary calcium on egg and

eggshell quality in Tegel and Leghorn pullets. Calcium retention, alkaline phosphatase and ATPase enzyme activity, intestinal mucosa morphometry and expression of calbindin D_{28k} will also be considered to provide a basic understanding of the principles involved in strain effects.

5.2. Materials and methods

Thirty-two (18 week old) Tegel (Red-Island red, female x Australope, male) were compared with 32 Leghorn (Leghorn, male x New Ham shire, female) pullets over four weeks. They were raised in the individual cages and fed under the same conditions as mentioned in the experiment 4. The effects of daily (5 pm) estrogen injection (E_2) at 0, 10, 50, 100 µg/kg body weight/day as well as low (2%) and high (4%) levels of dietary calcium were studied on these strains using a randomised complete block design with factorials (4 x 2 x 2 x 4). Dry matter, nutrient retention (CP, Ca, P, Mg, Na, and K) and growth, as described in chapter 2, were measured as was the main interaction effects of estrogen and calcium in both strains. Alkaline phosphatase, ATPase enzyme activity (Ca, Mg, Ca/Mg and Na/K), mucosa structure morphometry were measured as well as the expression of calbindinD_{28k} (based on methods elucidated in chapter 2).

Eggs were collected for 20 successive days at 4 pm. Egg and eggshell were weighed fresh. The first egg appeared in the second week of rearing in all treatment. The other egg and egg shell parameters were measured based on methods described in chapter 2. The composition of two diets in this experiment containing (high 4%, diet 1) and (low 2%, diet 2) calcium for 18- 22 week old Tegel and Leghorn pullets as recommended by NRC, (1994), were as follows:

Feed formulation for 18-22 week old Leghorn and Tegel pullets (diet composition g/100g)

Feed sources	Diet 1	Diet 2
1. Wheat	70.00	71.00
2. Barley	6.00	10.00
3. Meat meal (50% CP)	15.67	15.67
4. Lysine (synthetic)	0.25	0.25
5. Methionine (synthetic)	0.12	0.12
6. Marble (source of calcium)	2.25	7.25
7. Sunflower oil	0.38	0.38
8. Mineral, Vitamins & Yolk colorant	0.33	0.33
Total	**100.00**	**100.00**
Metabolisable energy (ME)[1]	11.68 MJ/kg	11.29
Crude protein[2]	16.13 %	16.64 %
Calcium [2]	4.00 %	2.15%
Total phosphorous[2]	0.93 %	0.95%
Fibre [2]	2.96 %	3.27%
Fat[2]	2.50%	2.60%

1, calculated; 2, measured; as a described in chapter 2.

The data were subjected to ANOVA and mean values compared with LSD.

5.3 Results

5.3.1 Growth and feed intake

Growth and feed intake are presented in the (Table 5.1). The initial body weight at 18 weeks of age of Tegel pullets (1.86 kg) was significantly greater (P<0.001) than Leghorn pullets (1.55 kg) but there was no significant differences between treatments (Ca and estrogen levels).

No significant difference occurred between the high and low levels of calcium in growth in terms of weight gain (WG) liver weight (LW), oviduct weight (OW), and shell gland (SG) weight in (g/100 g of body weight). A strain effect showed Leghorn pullets were significantly greater (P< 0.001) in OW and SG than Tegel (Table 5.1).

Body weight gain was reduced significantly (P<0. 001) with estrogen dosage.

Oviduct and shell gland weight were also reduced significantly ($P<0.01$) with 100 µg E_2/kg body weight/day compared with (0, 10 and 50 µg of E2), although no significant difference appeared in LW in this case (Table 5.1). SG weight showed a significant reduction (about 30%, $P<0.05$) response to the interaction both high and low calcium dietary and estrogen (100 µg /kg body weight /day) in comparison with other treatments (0, 10 and 50µg of E_2 at both low and high levels of calcium).

Daily feed intake was higher ($P<0.01$) in Tegel pullets than Leghorns, but feed intake per 100g of body and FCR were greater ($P<0.001$) and lower ($p<0.05$) respectively in Leghorn than Tegel. No significant interaction between different amount of dietary calcium and various levels of estrogen were observed on these factors in Tegel or Leghorn pullets (Table 5.1).

Table 5.1: The effect of calcium, strain and different level of estrogen on body characteristics and daily feed intake

Growth and feed intake	Calcium		Strain			Estrogen (µg /kg body weight/day)				
	H (4%)	L (2%)	Leghorn	Tegel	SEM	0	10	50	100	SEM
A. Growth										
Initial body weight (IBW, kg)	1.72	1.69	1.55^{b}	1.86^{a}	0.027	1.65	1.70	1.74	1.74	0.042
Body weight gain (WG, g)	232	224	244	212	19.20	323^{a}	158^{b}	203^{b}	227^{b}	27.20*
Liver weight (LW, g/100 BW)	2.78	2.64	2.76	2.65	0.086	2.66	2.69	2.75	2.74	0.12
Oviduct weight (OW, g/100g BW)	2.90	2.82	3.12^{a}	2.60^{b}	0.075	2.88^{ab}	2.93^{a}	3.04^{a}	2.60^{b}	0.11 *
Shell glands (SG, g/100g BW)	0.89	0.91	0.98^{a}	0.82^{b}	0.031	0.94^{a}	0.96^{a}	0.93^{a}	0.78^{b}	0.043*
B. Feed intake										
Daily feed intake (DFI, g)	100.50	97.70	96.00^{b}	102.10^{a}	2.07	94.80	99.90	102.80	98.80	2.93
Feed intake (FI, g/100g BW)	197.10	192.80	210.40^{a}	179.50^{b}	4.94	199.10	202.10	185.60	193.00	6.98
Feed conversion ratio (FCR, egg, g/ feed, g)	2.64	2.58	2.49^{b}	2.73^{a}	0.13	2.61	2.54	2.71	2.58	0.18

C. Sources of variation	Growth					Feed intake		
	IBW	BW G	LW	OW	SG	DFI	FI/100g	FCR
Ca	NS	NS	NS	NS	NS	NS	NS	NS
STR	****	NS	NS	****	****	**	****	*
E_2	NS	***	NS	**	**	NS	NS	NS
Ca x STR	NS	NS	NS	NS	NS	NS	NS	NS
Ca x E_2	NS	NS	NS	NS	*	NS	NS	NS
STR x E_2	NS	NS	NS	NS	NS	NS	NS	NS
Ca x STR x E_2	NS	NS	NS	NS	NS	NS	NS	NS

Mean Values on the same row without a common superscript are significantly different, * , (P<0.05), ** , (P<0.01), **** , (P<0.005), **** , (P<0.001) ;
SEM. standard error of mean; E2, estrogen; STR, strain; Ca, calcium; NS, not significant.

5.3.2 Nutrient retention

The results of nutrient retention are presented in the Table 5.2. On a low calcium diet DM and Ca retention were significantly greater ($P<0.001$) than on a high calcium diet. DM, Ca and Na retention were significantly greater ($P<0.01$) in Tegel than Leghorn, a similar trend occurred with CP ($P<0.001$). No significant differences were observed in P, K and Mg retention. With increasing estrogen doses, particularly at 50 and 100 μg E_2/kg body weight/day, retention of Mg decreased markedly ($p<0.01$). In contrast, no significant effects were observed with respect to estrogen on nutrients retention, although at the 10 μg of E_2 there was an increase in comparison with other levels of E_2 (0, 50 and 100 μg).

Interaction between low calcium and 10 μg of estrogen /kg body weight/day was significantly greater in the retention of CP ($P<0.005$), Ca ($P<0.01$) and K ($P<0.05$) compared with the other treatment groups. A significant increased effect ($P<0.01$) was identified between 50 μg of E_2 and a high level of calcium on Na retention. No significant differences were found in the interaction of different levels of dietary calcium and the various levels of estrogen on, P, Mg retention (Fig 5.1) and. DM.

In terms of interaction between strain (STR) and calcium dietary (STR x Ca) on nutrients retention, significant interaction between Leghorn pullets and low dietary calcium ($P<0.05$) and Tegel with high dietary calcium ($P<0.001$) were observed for K and Mg retention respectively. The interaction of high Ca x strain x 50 μg E_2 on CP and Ca ($P<0.05$) and Na ($P<0.01$) retentions was significantly higher in Leghorn than Tegel pullets.

Table 5.2: **Nutrient retention in response to calcium, strain of pullets and estrogen levels**

	Calcium		Strain			Estrogen (μg /Kg body weight/day)				
	H (4%)	L (2%)	Leghorn	Tegel	SEM	0	10	50	100	SEM
A. Nutrients retention (%)										
Dry matter (DM)	73.24^{b}	78.23^{a}	73.86^{b}	77.6^{a}	0.91	76.50	76.26	75.38	74.81	1.28
Crude protein (CP)	86.59	67.73	85.76^{b}	88.55^{a}	0.50	86.50	88.16	87.12	86.84	0.71
Calcium (Ca)	31.90^{b}	44.50^{a}	34.30^{b}	42.10^{a}	2.10	38.80	40.70	37.60	35.70	2.97
Phosphorus (P)	15.50	15.10	15.10	15.50	2.01	14.00	21.70	16.40	9.00	2.84
Sodium (Na)	42.20	42.00	39.20^{b}	50.00^{a}	2.93	41.90	48.80	48.60	39.10	3.38
Potassium (K)	23.60	28.80	23.70	28.70	2.27	25.80	33.70	22.40	22.90	3.22
Magnesium (Mg)	18.00	21.00	17.00	22.00	2.06	21.40^{ab}	26.80^{a}	12.90^{b}	16.90^{b}	2.91*
B. Sources of variation	**DM**	**CP**	**Ca**	**P**	**Na**	**K**	**Mg**			
Ca	****	NS	****	NS	NS	NS	NS			
STR	**	****	**	NS	**	NS	NS			
E_2	NS	NS	NS	NS	NS	NS	**			
Ca x STR	NS	NS	NS	NS	NS	*	****			
Ca x E_2	NS	***	**	NS	**	*	NS			
STR x E_2	NS	NS	NS	NS	NS	NS	NS			
Ca x STR x E_2	NS	*	*	NS	**	NS	NS			

Mean Values on the same row without a common superscript are significantly different, * , ($P<0.05$), ** , ($P<0.01$), **** , ($P<0.005$), **** , ($P<0.001$);SEM, standard error of mean; E_2, estrogen; STR, strain; NS, not significant.

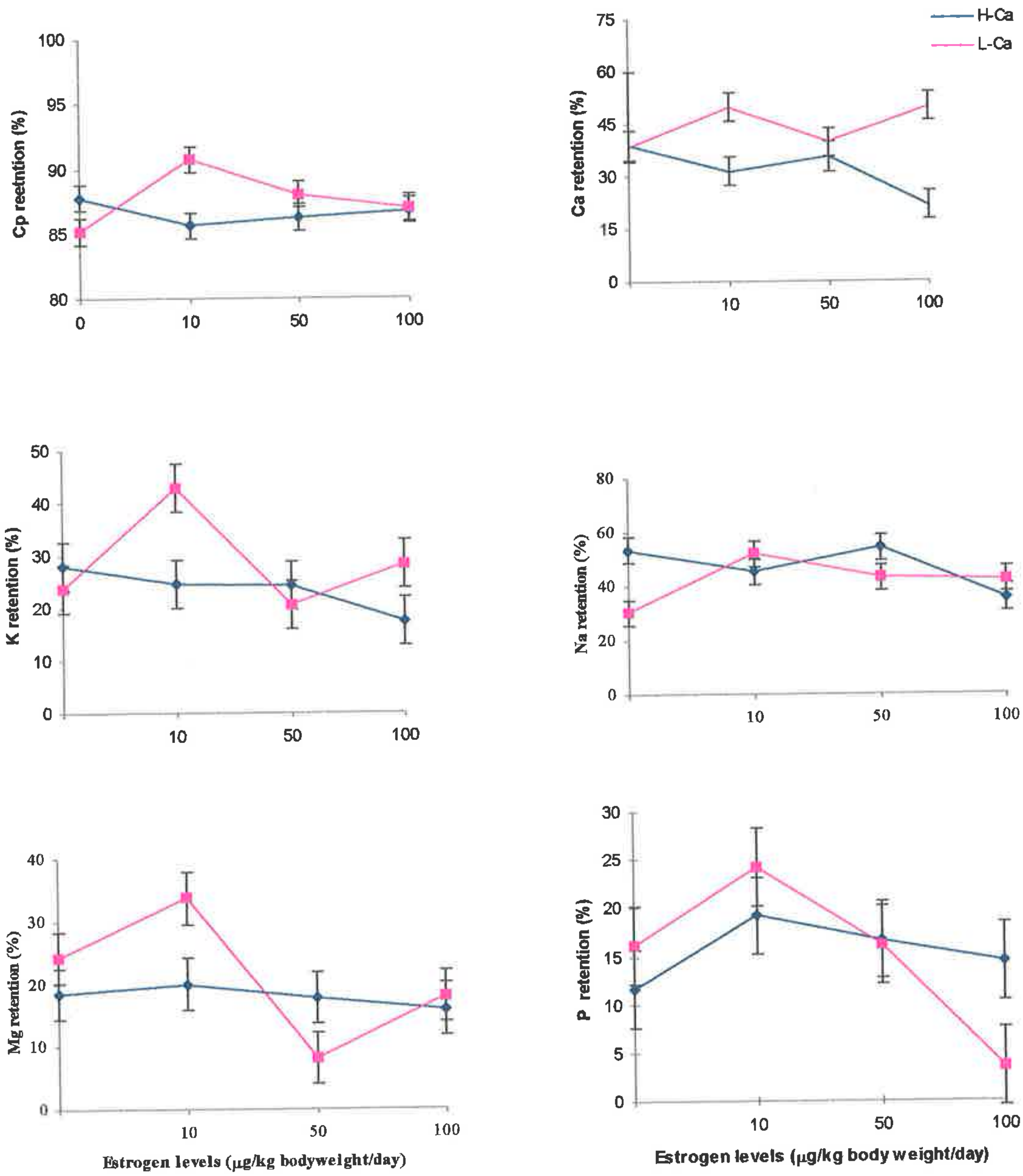


Figure 5. 1: The effect of calcium and estrogen interaction on the retention of nutrients.

5.3.3. Egg and eggshell characteristics

5.3.3.1. Egg characteristics

Egg and eggshell characteristics are represented in Table 5.3. Compared with the low calcium diet, the high calcium diet caused no significant differences in egg production (EP), egg mass (EM) and specific gravity (ESG), but egg weight (EW) was significantly greater ($P<0.05$) on high calcium (Table5.3)

The Tegel pullets had significantly greater EP ($P<0.001$) and EM ($P<0.01$) than Leghorn pullets. In contrast, Leghorn pullets had significantly higher ($P<0.01$) ESG than Tegel, although EW was similar in the both strains (Table 5.3).

Similar effects were observed for most egg characteristics obtained from hens maintained different levels of estrogen (0 to 100 µg/kg body weight/day, Table 5.3). The exceptions were found in EP and EM which were significantly lower ($P<0.05$) at 0 µg of E_2 than other levels of estrogen, 10, 50 and 100. EW was significantly lower ($P<0.05$) at 100 µg of E_2 than 0, 10 and 50 µg. No changes were found in ESG with respect to different estrogen levels (Table 5.3 A).

The interaction of calcium and estrogen on EW ($P<0.005$) was significantly greater at 50 µg/kg body weight /day of estrogen and high levels of calcium (Fig 5.2). While the interaction between strain (STR), Leghorn pullets and high calcium dietary on ESG was significant ($P<0.05$), no differences were observed in EP, EM and ESG by interaction between the other treatments.

5.3.3.2. Egg shell characteristics

Shell weight increased (P<0.05) at the high level of calcium compared with the low calcium level (Table 5.3). Although there were no significant effects between high and low levels of calcium on ST, SP and WU of the shell, all of these parameters were greater at high levels of calcium with exception in SFA, of shell which was significantly greater (P<0.05) in this case (Table 5.3. B).

Eggshell quality including SW and SP (P<0.05) and shell weight per unit area (P<0.01) were significantly greater in Leghorn pullets than in Tegel pullets (Table 5.3 B). No significant response was shown in ST and SFA in regard to strain (Table 5.3 B).

Although no significant effects appeared in SW, SP and WU with respect to varying levels of estrogen, greater significant differences were found in ST (P<0.001) at 0 and 50 and in SFA (P<0.05) at 0, 10 and 50 compared with the other level of estrogen treatments (Table. 5.3 B).

In terms of interaction effects on shell quality, SW (P<0.001), WU (P<0.05), SFA and ST (P<0.01) were significantly greater by interaction between 50 µg/kg body weight /day of estrogen and high levels of calcium (Fig 5.2) and also (Table 5.3). No differences were found by interaction between different dietary calcium and various levels of estrogen on SP.

No responses were identified in terms of interaction between strains, STR (Leghorn and Tegel pullets) and different levels of estrogen, E_2, 0, 10, 50 and 100 μg (STR x E_2) on shell characteristics. The same trend has been shown in regard to interaction between strain and various dietary calcium, high and low (STR x Ca) on shell characteristics. Although no significant differences were recognised in the interactions between calcium, estrogen and strains (Ca x E_2 x STR), on shell quality, it appeared that shell thickness (411.2, μm) and shell weight (5.51, g) were greater about 23μm and 0.5 g respectively in Leghorns at high calcium and 50 μg E_2 /kg body weight than in Tegel pullets.

Table 5.3: Calcium, strain and different levels of estrogen on egg and eggshell characteristics

	Calcium		Strain			Estrogen (µg /kg body weight/day)				
Egg and egg shell characteristics	H (4%)	L (2%)	Leghorn	Tegel	SEM	0	10	50	100	SEM
A. Egg characteristics										
Production (EP, egg/hen/house %)	52.30	61.50	48.7[b]	65.10[a]	3.91	43.40[b]	62.10[a]	62.00[a]	60.00[a]	5.53
Weight (EW, g)	49.61[a]	47.38[b]	48.66	48.34	0.74	47.57[a]	50.00[a]	50.18[a]	46.24[b]	1.05
Mass (EM, g/day)	26.30	29.70	23.60[b]	32.30[a]	1.91	20.20[b]	30.80[a]	30.80[a]	30.00[a]	2.70
Specific gravity (ESG)	1.0861	1.0856	1.0870[a]	1.0846[b]	0.0006	1.0853	1.0857	1.0856	1.0867	0.0009
B. Egg shell characteristics										
Weight (SW, g)	4.89[a]	4.62[b]	4.88[a]	4.64[b]	0.08	4.65	4.89	4.88	4.62	0.11
Thickness (ST, µm)	362.80	357.40	364.10	356.10	4.15	375.20[a]	340.80[b]	385.80[a]	338.70[b]	5.87
Surface area (SFA, cm^2)	61.31[a]	59.45[b]	60.51	60.26	0.60	59.71[ab]	61.55[a]	61.70[a]	58.56[b]	0.84
Percentage (SP, %)	10.00	9.78	10.15[a]	9.64[b]	0.15	9.73	10.07	9.76	10.10	0.21
Weight per unit area (WU, mg/ cm^2)	79.73	77.61	80.43[a]	76.90[b]	0.90	77.67	79.37	78.74	78.89	1.27
	Egg characteristics					Egg shell characteristics				
C. Sources of variation	EP	EW	EM	ESG		SW	ST	SFA	SP	WU
Ca	NS	*	NS	NS		*	NS	*	NS	NS
STR	***	NS	**	**		*	NS	NS	*	**
E_2	*	*	*	NS		NS	****	*	NS	NS
Ca x STR	NS	NS	NS	NS		NS	NS	NS	NS	NS
Ca x E_2	NS	***	NS	NS		****	**	**	NS	*
STR x E_2	NS	NS	NS	*		NS	NS	NS	NS	NS
Ca x STR x E_2	NS	NS	NS	NS		NS	NS	NS	NS	NS

Mean Values on the same row without a common superscript are significantly different, * , (P<0.05), ** ,.(P<0.01), **** ,.(P<0.005), ****, (P<0.001); SEM, standard error of mean; E_2, estrogen; STR, strain; Ca, calcium; NS, not significant

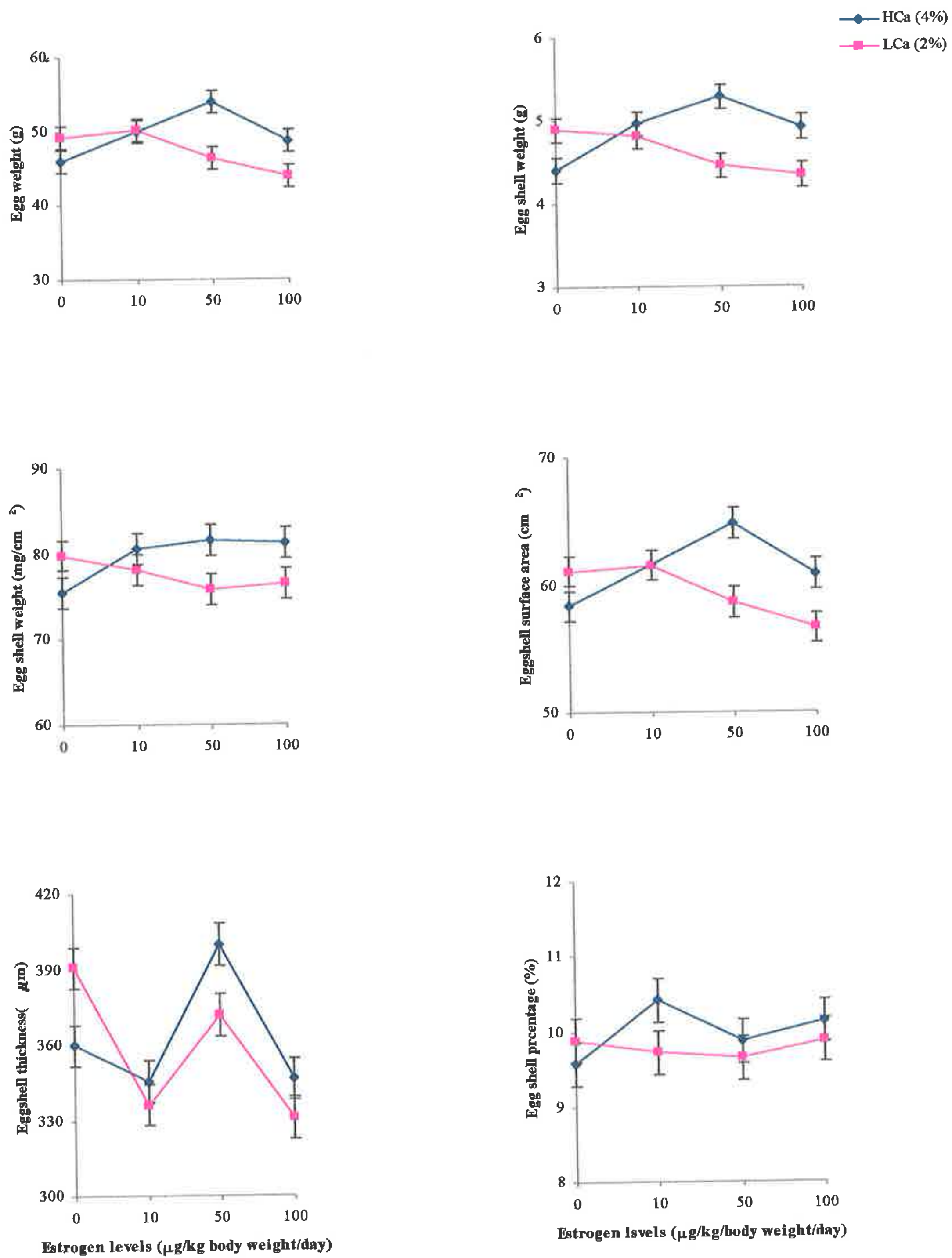


Figure 5.2: Interaction of different levels of calcium and estrogen on egg and eggshell characteristics.

5.3.4. The intestinal mucosa morphometry

The 50 µg of E_2 showed more response on eggshell quality, based on this point 0 and 50 µg of E_2 treatment group have been compared for mucosa structure. The morphometry of different regions of the small intestine is presented in Table 5.4. Crypt depth was significantly increased ($P<0.01$) in jejunum tissue at the high calcium diet. There were no significant changes in the duodenum, and ileum crypt depth between the high and low calcium diets. No differences was observed in the crypt depth in the duodenum, jejunum and ileum with respect to strain and 0 and 50 µg E_2/kg body weight/day.

Villus height was similar in the three regions of the small intestine with respect to dietary calcium in both Leghorn and Tegel pullets and at 0 and 50 µg E_2/kg body weight/day. The villus surface area of the ileum in the Tegel pullets was significantly ($P<0.05$) larger than in Leghorn pullets (Table 5.4). No significant effects were apparent in all the interactions between calcium, estrogen and strain in mucosa morphometry, with the exception of the interaction between calcium and strain (Tegel and high level of calcium) and also the interaction between estrogen and calcium (50 µg of E_2 and low calcium) which were significantly greater (about 0.054 mm^2, $P<0.05$) and (about 0.067 mm^2, $P<0.01$), on jejunal surface area, respectively than in the other treatment groups (Table 5.4).

Table 5.4: Intestinal morphometry regarding calcium, strain and estrogen levels

Segments	Calcium		Strain		Estrogen		
	H (4%)	L (2%)	Leghorn	Tegel	0	50	SEM
A. Crypt dept (CR, µm)							
Duodenum (D)	346.40	337.00	334.00	349.50	334.90	348.60	10.14
Jejunum (J)	316.90^{a}	289.7^{b}	305.00	301.70	305.90	300.70	6.56
Ileum (L)	252.30	242.30	238.00	256.70	251.40	243.30	7.82
B. Villus height (VH, µm)							
Duodenum	2430.00	2490.00	2458.00	2462.00	2459.00	2461.00	83.80
Jejunum	2182.00	2128.00	2123.00	2186.00	2122.00	2187.00	49.60
Ileum	1336.00	1305.00	1262.00	1379.00	1290.00	1351.00	43.30
C. Villus surface area (VSF, mm^2)							
Duodenum	0.675	0.659	0.649	0.686	0.660	0.674	0.033
Jejunum	0.509	0.498	0.498	0.510	0.497	0.511	0.014
Ileum	0.313	0.303	0.284^{b}	0.332^{a}	0.304	0.312	0.015

D. Source of variation	Crypt depth			Villas height			Villas surface area		
	DCR	JCR	LCR	DVH	JVH	LVH	DVSF	JVSF	LVSF
Ca	NS	**	NS	NS	NS	NS	NS	NS	NS
STR	NS	NS	NS	NS	NS	NS	NS	NS	*
E_2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Ca x STR	NS	NS	NS	NS	NS	NS	NS	*	NS
Ca x E_2	NS	NS	NS	NS	NS	NS	NS	**	NS
R x E_2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Ca x STR x E_2	NS	NS	NS	NS	NS	NS	NS	NS	NS

Mean Values on the same row without a common superscript are significantly different, * , ($P<0.05$), ** , ($P<0.01$); E_2, estrogen (µg /kg body weight/day); SEM, standard error of mean; STR, strain; Ca, calcium; NS, not significant.

5.3.5. Enzyme activity

Due to the greater response of shell quality to the 50 µg E_2 treatment enzyme activities have been assessed in this and control groups only. Alkaline phosphatase and ATPase activity are presented in the Table 5.5. This table shows that there were no significant effects due to calcium (high and low levels), strain (Leghorn and Tegel) and estrogen levels (0 and 50 µg), on alkaline phosphatase, Ca, Mg, Ca/Mg and Na/K ATPase activity in the jejunal homogenate.

No differences appeared in the interaction between different dietary calcium, different levels of estrogen and strain (Ca x $_{E2}$ x STR) on Ca, Mg and Ca/Mg and ATPase enzymes activity with exception in alkaline phosphatase and Na/K ATPase. Since there was a significant interaction ($p<0.05$) between Tegel pullets, high dietary calcium and 50 µg of E_2 (Tegel x high Ca x 50 µg of E_2) on alkaline phosphatase compared with other treatment groups. Also a significant interaction ($P<0.01$) between Tegel pullets, low level of calcium at the 50 µg of E_2, (Tegel x low Ca x 50µg of E_2) on activity of Na/K was observed in comparison with other treatments. Although no significant differences were recognised in the interaction of (Ca x E_2 x STR) on the most ATPase enzyme activities, it seems the activities of these enzymes were higher at 50 µg of E_2 in Tegel pullets and high levels of calcium compared with other treatments.

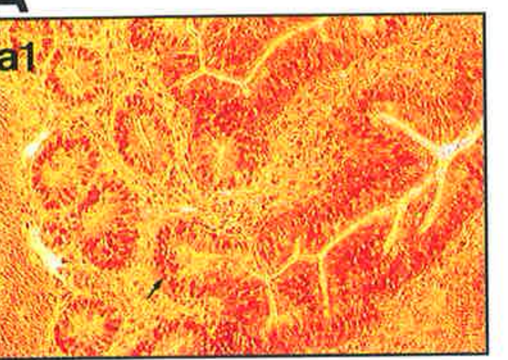
5.3.6. Calcium binding protein localisation.

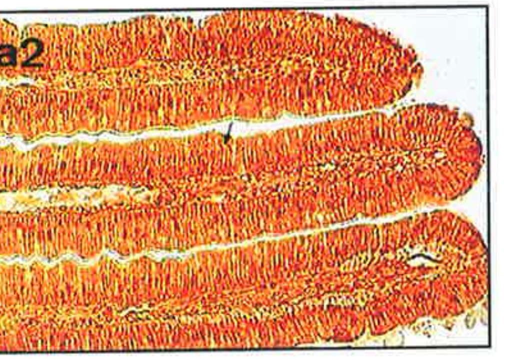
The expression of calbindin D_{28k} was examined in 0 and 50 µg E_2/kg body weight/day treatment group, since maximum differences in shell quality was observed between

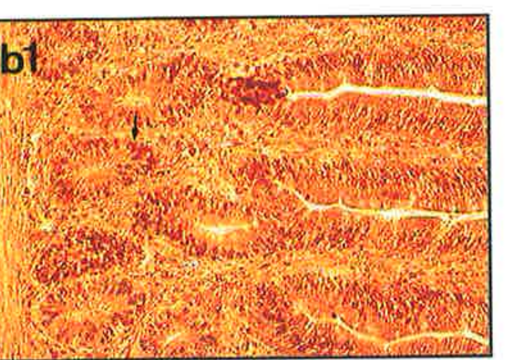
those groups. The expression of the calcium binding protein (calbindin D_{28k}) in ~~the~~ all treatments is shown in Plate 5.1. No differences were seen in the interaction of high and low calcium at different levels of estrogen (0 and 50 μg/kg body weight/day) in both Leghorn and Tegel pullets (Plate 5.1). The expression of calbindin D_{28k} was localised to both the cytoplasm and nucleus of ~~the~~ the absorptive epithelial cells.

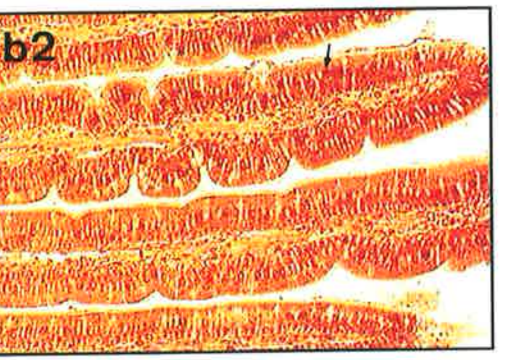
Plate 5.1: Immunohistochemical localisation of calbindin D28K in crypt (1) and villus (2) regions of typical jejunal tissue taken from 22 week old Leghorn (A) and Tegel (B) pullets in response to low (2%) or high (4%) dietary calcium and intramuscular injection of either 0 or 50 μg estrogen/ kg body weight/day. Panels a and b represent tissue taken from hens treated with 0 μg E_2, and maintained on low and high calcium respectively. The effect of exogenous estrogen, 50 μg E_2, for the same dietary treatments are represented in panels c and d.. Arrows indicate positive reactions in epithelial cells. Typical negative controls are represented in panels labelled with n.

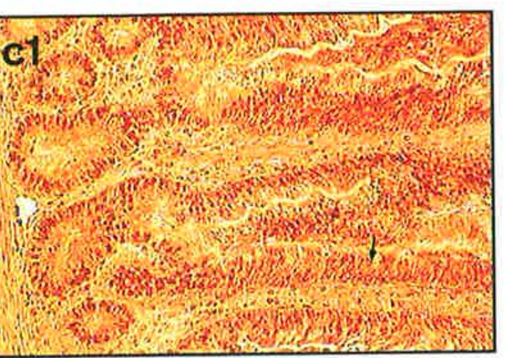
A

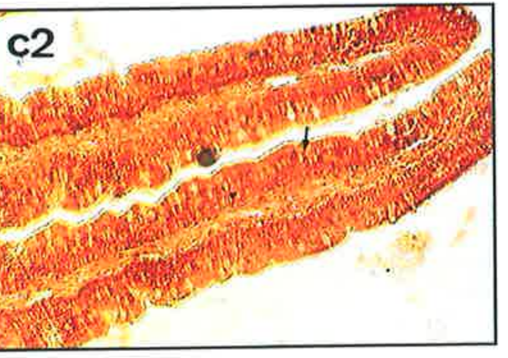


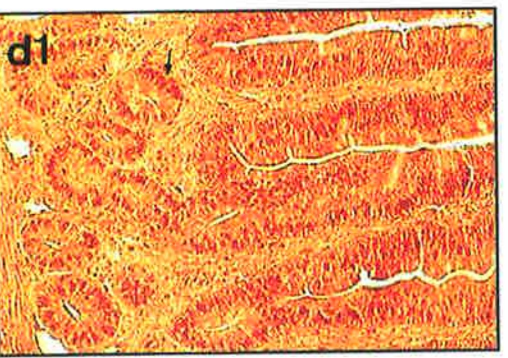


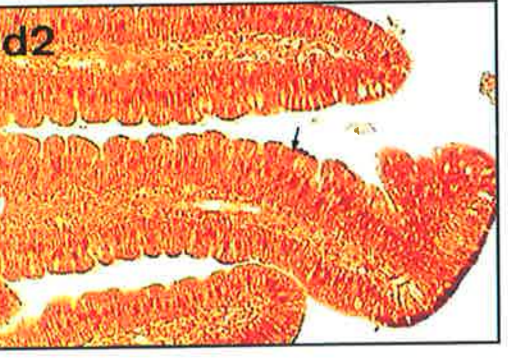


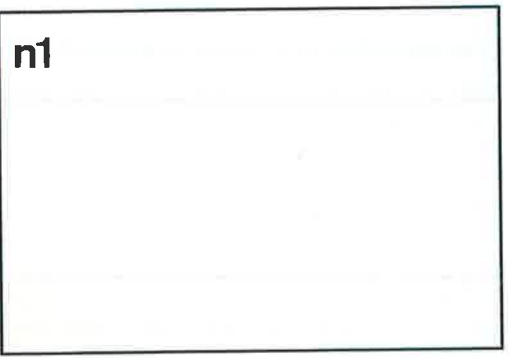


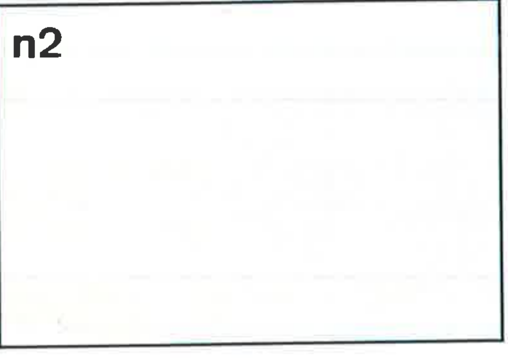




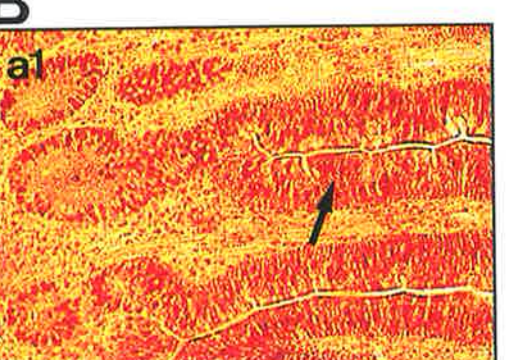


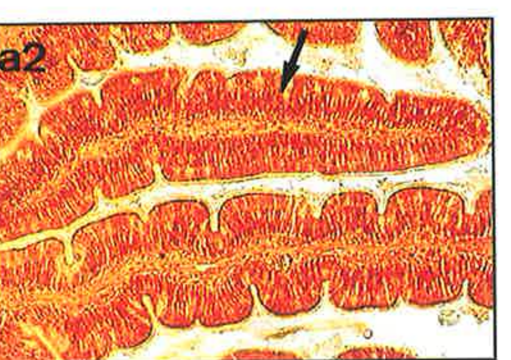


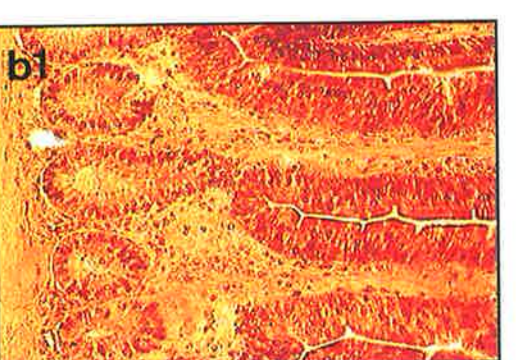


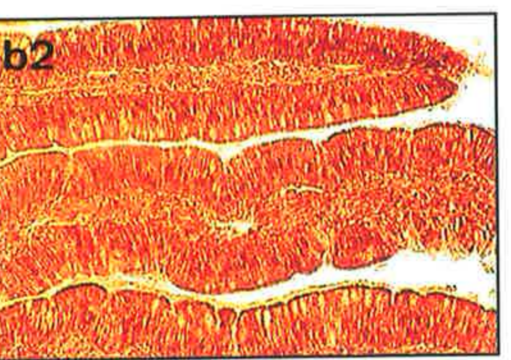


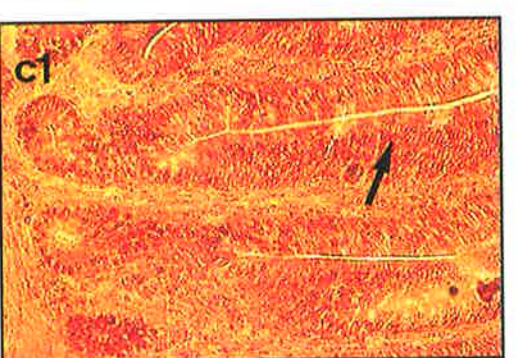
B

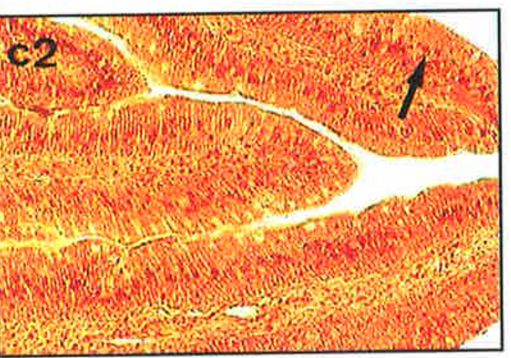


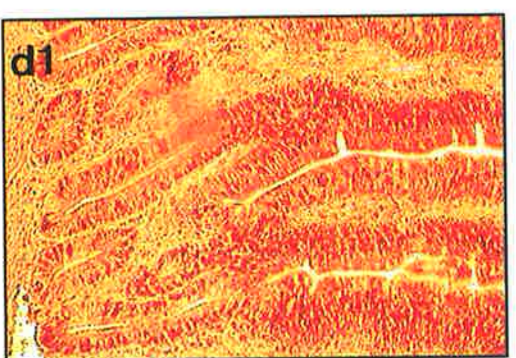


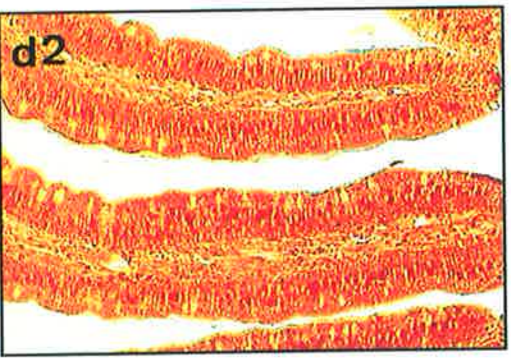












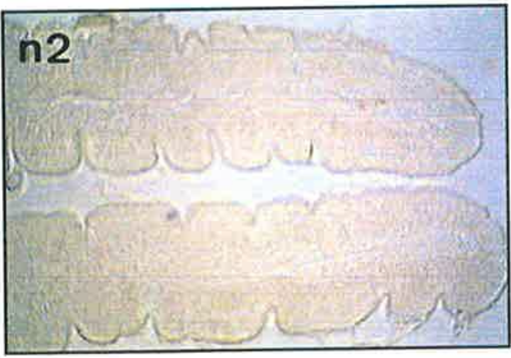


Table 5.5: Enzyme activities in homogenate of Jejunum from 22 week old Leghorn and Tegel pullets, responseto interaction of estrogen and calcium.

		Leghorn		Tegel		
Enzymes activities	**Ca (%)/ estrogen[1]**	**0**	**50**	**0**	**50**	**SEM[2]**
Alkaline phosphatase[3]	4	8.80	8.60	12.20	26.0	6.50
	2	9.50	15.7	10.20	9.30	6.50
Ca ATPase[4]	4	2.72	3.73	2.70	4.16	0.49
	2	2.41	3.52	2.81	1.99	0.49
Ma ATPase	4	2.36	3.79	2.49	4.21	0.68
	2	2.24	2.98	2.52	1.87	0.68
Ca/Mg ATPase	4	2.36	2.90	2.38	4.23	0.51
	2	2.27	3.01	2.51	1.78	0.51
Na/K ATPase	4	0.318	0.328	ND[5]	0.408	0.09
	2	0.309	ND	0.388	0.503	0.09
Sources of variation		**Alk. Phos.**	**Ca.**	**Mg**	**Ca/Mg**	**Na/K**
Ca		NS	NS	NS	NS	NS
STR		NS	NS	NS	NS	NS
E_2		NS	NS	NS	NS	NS
Ca x STR		NS	NS	NS	NS	**
Ca x E_2		NS	NS	NS	NS	NS
STR x E_2		NS	NS	NS	NS	**
Ca x E_2 x STR		*	NS	NS	NS	**

Mean Value on the same row without a common superscript are significantly different, * , (P<0.05), ** , (P<0.01); 1 .estrogen, µg kg body weight/day; 2. SEM, standard error of mean; 3. µmol/ mg protein/ min; 4. µg Pi product/mg protein/ min.; NS, not significant; ND, no detected; Alk. Phos, alkaline phosphatase; Ca, CaATPase; Mg, MgATPase; Ca/Mg, Ca/Mg ATPase; Na/K, Na/K ATPase.

5.4. Discussion

5.4.1. Growth and feed efficiency

In this experiment over all, growth and feed efficiency were not changed predominantly by estrogen or calcium levels, although some reductions were observed in growth and feed efficiency at high estrogen dose (100 µg E_2) due to strain (Leghorn and Tegel).

Differences in initial body weight were likely to be a reflection of the genetic potential of specific strains. Increasing estrogen dose resulted in reducing (P<0.001) weight gain. This results have been emphasised by Sommerville *et al* (1989). In agreement with Qin and Klandrof (1995) results, the oviduct and shell gland weight per 100 g of body weight were increased by estrogen doses (10, and 50 µg E_2) but no significant effects were found in these aspects. In contrast at a high level of estrogen (100µg E_2) significant reductions (P<0.01) were found in oviduct and shell gland weight. This could be related to the fact that high estrogen doses have an adverse effect on the reproductive system (Lien and Cain, 1987).

Results from the current experiment showed that different strains have a different response in oviduct and shell gland weight. Hence Leghorn pullets were significantly greater (P<0.001) than Tegel in these aspects. This has not been identified in literature previously.

Daily feed intake is an important factor in animal production. There is evidence that feed intake of the hen may vary during the day to regulate her intake of calcium in

relation to shell formation (Gilbert, 1983). Calcium intake is correlated with Ca retention, which may change egg and eggshell quality and also enzyme activity. Thus feed intake is an important measurement. In regard to initial weight, daily feed intake of Tegel pullets was significantly greater ($P<0.01$) than that of Leghorn pullets. However, feed intake (g/100g of body weight) and feed conversion in Leghorn pullets were significantly higher than for Tegel pullets. Since the initial weight of Leghorn pullets was lower than Tegel pullets this situation induced Leghorn pullets to grow faster while improving their FCR. Thus, they are not only different in genetic potential, but also the energy and nutrients requirement for maintenance of Tegel pullets (with greater initial weight) are larger than that of Leghorn pullets.(Hunton, 1995). Therefore the FCR of egg production in this case was improved in Leghorn compared with Tegel. Numerous studies and the current experimental results suggest there is no significant effect of estrogen treatments on feed intake, although feed intake may be depressed by high estrogen dosage (Adams *et al.*, 1950; Robey *et al.*, 1988).

5.4.2. Nutrient retention

The amount of calcium retained by the hen is a major consideration in the supply of calcium. Many factors affect its retention, for example, crop size, eating time and the requirement for essential nutritional factors (Hunton, 1995). The retention of dry matter (DM) which includes many nutrients, also can affect calcium retention. In addition, P and Mg supply can contribute to calcium retention and enzyme activity. Sodium as a carrier through a Na/Ca exchange mechanism (Civitelli and Avioli, 1994) and also in company with potassium as Na/K ATPase enzyme may effect calcium absorption (Van Os, 1987)

As demonstrated in a previous study (Petersen, 1965), data in the present study indicates that the retention of dry matter and Ca in low level calcium diets were significantly greater ($P<0.001$) than in high Ca diets. DM and all mineral retentions were greater in the Tegel pullets. Others have not reported this for this particular situation. In turn, CP ($P<0.001$), Ca and Na ($P<0.01$) were identified as being affected by dietary treatments.

Although Mg showed a significant response ($P<0.01$) to 10 µg of E_2, compared with 50 and 100 µg of E_2, it was similar to 0 µg of E_2. Therefore, it seems that there is no significant effect on retention of most nutrients by different levels of estrogen, but a trend to higher nutrient retentions in the 10 µg of E_2 group was observed. No reported has been identified in this matter.

The interaction effect of Ca levels, different levels of estrogen, E_2 and strains, STR (Ca x E_2 x STR) on CP, Ca and Na retention were significantly higher at high level of calcium, 50 µg of E_2 and in Leghorn pullets compared with Tegel pullets. This might be related to the advance maturation in Leghorn pullets.

5.4.3. Egg and eggshell quality

Evidence has shown that more than 50% of variation in eggshell quality is dependent on non-genetic factors, because the heritability of shell quality is 30-40% (Hunton, 1995). In addition, a decrease in the average egg weight has usually been associated with selection for improving eggshell quality in commercial breeding (Hunton, 1987; Hunton, 1995). This information has lead to the consideration of number of

nutritional and biochemical factors which may influence egg and shell quality. Calcium is a major nutrient that may have a priority role on egg and eggshell quality.

Findings of this study show that egg and eggshell quality are very sensitive to the calcium concentration in diets, but this is strain specific and depends on E_2 levels. As shown in a previous studies (Scott, 1982 and Huton, 1995) egg weight increases dramatically by high levels of calcium. In the present study egg production and egg mass were greater in Tegel than Leghorn pullets. In contrast, egg weight and specific gravity, which are correlated to each other, were higher for Leghorns. Egg characteristics have not examined in Tegels pullets in such particular case. In most estrogen treatments, egg production and egg characteristics show similar trends (Lien and Cain, 1987), with the exception in egg production and egg mass in the present study at 0 and 100 µg of E_2, which were lower than 10 and 50 µg of E_2.

The results of this experiment have shown that all shell characteristics improved at high levels of calcium. A particularly significant effect was seen in shell weight, compared with low levels of calcium. Similarly significant effects were observed between Leghorn and Tegel for SW, SP and shell per unit area (UW). Although comparison between Leghorn and Tegel pullets have not reported, but in general, the improvement on shell quality at a high level of calcium was also noted by Taylor *et al.* (1995).

It seems that 50 µg of E_2 improved shell thickness and surface area of the shell, although no significant differences were identified between this level of estrogen and 0 µg of E_2. This is particularly true for hens maintain on diets low in calcium (Fig. 5.2) Similar results were observed by (Qin and Klandorf, 1995). The reason for this

physiological reaction could be related to the effect of interaction between of PTH and vitamin D_3 (1,25$(OH)_2D_3$) on bone and kidney calcium reabsorption, since they are induced by low levels of calcium in the plasma (Brody, 1994). Vitamin D_3 (1,25$(OH)_2D_3$) can be produced in the kidney by the effect of the PTH and endogenous estrogen. Consequently the production of vitamin D_3 (1,25$(OH)_2D_3$) enhance calcium absorption in the small intestine and finally increases calcium deposition in the shell thus improving the thickness and surface area of shell at the low level of estrogen (0 µg).

5.4.4. Intestine morphometry

Increasing the nutrient absorption may be related to the structure of the intestinal villi. Hurwitz *et al.* (1971) and Hurwitz, (1989) reported that the main absorption of calcium occurred in the jejunum. This was also considered in the current study. The 50 µg of estrogen showed a larger effect on shell weight and shell thickness compared with the other estrogen levels. Therefore at this level of estrogen it appeared more calcium was deposited in the shell. For this reason, this level of estrogen (50 µg) was chosen to be tested in morphometry of the intestine and also in enzyme activity. Crypt depth in the jejunum was significantly greater ($P<0.01$) at high level of calcium. No significant differences with respect of strain effect was found in different regions of the intestine, with the exception of villus surface in the ileum of Tegels, which was larger ($P<0.05$) than in Leghorn pullets. No differences were identified in different levels of estrogen in the intestinal regions. No previous report has been issued with respect to estrogen levels on intestine regions. Overall most of the intestinal regions were increased by high dietary calcium, Tegel pullets and high level of estrogen effects (50 µg E_2). These results, particularly those of

increased an intestinal morphometry in Tegel pullets, may be coupled to an enhanced of calcium retention.

5.4.5. Enzyme activity

Intestinal calcium transport is positively related to the enzyme activity (Ca ATPase and alkaline phosphatase) because the main mechanism for calcium transport through the plasma membrane is active transport (Bronner, 1992). Active transport needs to be supported by energy produced by phosphorylation during the ATP cycle. The activity of one enzyme in the ATP cycle, ATPase are an important factor in producing the energy required for calcium transport through plasma membranes. In this study the effect of Ca ATPase, Mg ATPase and Ca/Mg ATPase on calcium absorption were assessed. Calcium exchange with Na and Mg ATPase are affected by Na/K ATPase so this activity also needed to be considered. Alkaline phosphatase may be induced by vitamin D_3 ($1,25(OH)_2 D_3$), (Nys and Laage, 1984) which has major role on calcium transport. For this reason, the activity of alkaline phosphatase in the jejunal homogenate was also determined.

The jejunal homogenate was chosen for assays of enzyme activity because Grunder and Tsang (1984) and Hurwitz (1989) reported that the upper jejunum was the major site for calcium absorption. The results of the current study (Table 5.5) show that although there were no significant interactions between dietary calcium (2 and 4%), estrogen levels (0 and 50 μg E_2) and strain on Ca, Mg and Ca/Mg ATPase activity, significant ($P<0.01$) interaction of (Tegel x 50μg E_2 x Low Ca) was observed on Na/K ATPase activity. Alkaline phosphatase activity was also significantly increased ($P<0.05$) by interaction of (Tegel x 50 μg E_2 x high Ca). Overall the

activity of most enzymes including alkaline phosphatase, Ca, Mg, Ca/Mg and Na/K ATPase were greater with the interaction of 50 µg of E_2, high level of calcium and Tegel were greater than other treatment groups. Nys and Laage (1984) have reported similar findings.

5.4.6. Expression of calcium binding protein (calbindin D_{28k})

The calcium binding protein CaBP (calbindin D_{28k}) is the other principal factor involved in calcium absorption during the egg production in laying hens. The same treatments levels (at 0 and 50 µg of E_2) for assay of calbindin.D_{28k} was chosen for this experiment. No differences in calbindin $_{D28k}$ level was detected between treatments. The effect of estrogen and different amount of calcium on calbindin D_{28k} levels in different strains has not been reported previously. The expression of calbindin D_{28k} was localised in the absorptive cells and in epithelial cells in the villus of the small intestine as described by Jande *et al.* (1981).

5.4.7. Conclusion

The results of this experiment demonstrated that Leghorn pullets are more responsive to factors effecting their eggshell quality than Tegel pullets, although their egg characteristics and calcium retention were poorer than Tegel pullets. The question arising from these observations is, what is the relevance of calcium, strain (Leghorn), estrogen and their interaction in response to shell quality? The results suggest the interaction between estrogen and calcium may mediate other physiological responses such as parathyroid hormone (PTH) which may play a vital role in decreased calcium retention and low Ca ATPase activity in Leghorn pullets. Therefore at low levels of plasma calcium, PTH may affect the bone calcium reabsorption and stimulate renal

1-hydroxlase activity which converts $25(OH)_2D_3$ to $1, 25(OH)_2D_3$.

The physiological increased enzyme activities in this experiment may have improved the calcium retention in Tegel pullets in comparison with Leghorn. In contrast increased shell quality (SW, ST, SFA, SP and WU) in Leghorns compared with Tegels with less calcium retention, low enzyme activities and decreased intestinal regions morphometry may be related to a more effective interaction between PTH and vitamin D_3 ($1,25(OH)_2D_3$) for calcium reabsorption. The higher genetic potential of Leghorns compared with Tegels in these terms may be relevant. Intracellular calcium transport which is associated with contribution between several organelles, such as mitochondria, golgi, endoplasmic reticulum and also their action on calcium transport inside the cells and also across basolateral membranes could be the another reason for this particular reaction of Leghorn pullets. The ability of Leghorn pullets to produce calcium carbonate which is associated by carbonic anhydrase enzyme activity in shell glands, may be higher in this strain than in Tegel. This could be the reason for deposition of calcium carbonate in the shell, after the intestinal calcium absorption (Petersen, 1965; Hurwitz, 1989).

PTH also controls calcium metabolism in a complex relationship between the kidney, shell glands, bone, small intestine and blood during the shell formation. Since the steady-state of calcium concentration in the blood for synthesis of calcium carbonate during the shell formation must be maintained by PTH action on bone and kidney associated with estrogen to produce the vitamin D_3. These relationships may be more efficient in Leghorn than Tegel pullets. The genetic potential of those pullets might be greater for induction of calcium absorption from the shell gland which consequently affects the eggshell characteristics.

Results showed that, not only shell characteristics (shell weight, surface area of shell) were significantly ($P<0.05$) greater at high level of calcium, but egg weight also was improved remarkably ($P<0.05$) by high level of calcium compared with low level, as emphasised by Scott *et al.* (1982) and Gilbert (1983).

Although the interaction of estrogen and calcium at different levels were tested in this experiment, the interaction between 50 µg E_2 /kg body weight /day with high level of calcium was the most sensitive. Therefore with respect to shell quality Leghorn pullets were more sensitive to this level of estrogen (50 µg E_2) at high levels of calcium.

In conclusion, over all no significant response was observed in regard to estrogen treatments compared with control group. Thus the normal physiological level of estrogen was sufficient to show the full capacity of their reproductive system. However in the interaction between strain, estrogen and dietary calcium, a greater physiological response was found in Leghorn pullets in regard to shell quality. Such a response appeared in the interaction of (high Ca x Leghorn x 50 µg of E_2) in SW (5.51, g) and ST (411.2) of Leghorn pullets which were greater (0.5 g and 23 µm respectively) when compared with the others treatment groups. These findings demonstrated that Leghorn pullets (with a greater response to dietary calcium and level of estrogen would be the most useful strain in the next experiment in testing a plant estrogen (e.g. from soybean) as a dietary estrogen in laying hens.

CHAPTER SIX

INTESTINAL FUNCTION OF 10-14 WEEK OLD LEGHORN PULLETS IN RESPONSE TO EXOGENOUS ESTROGEN AND A HIGH PHYTOESTROGEN DIET

"large amounts of phytoestrogen have been found in soybean meal"
(Baghurst, 1997)

6.1. Introduction

Estrogenic compounds (phytoestrogens) have been identified in forages. They have been shown to have deleterious effects on reproduction in several species of mammals (Lien *et al.*, 1985).

Plant compounds with estrogenic activity may play an important role in the reproductive activity of laying hens particularly in pullets during the development of their reproduction system (Lien *et al.*, 1985; Lien and Cain, 1987). Although phytoestrogens are found in a wide variety of plants, most have only weak estrogenic activity. However, two potent phytoestrogens are present in soybean which is frequently a major feed ingredient in poultry feed. These are the phytoestrogens genistein and daidzein. McDonald *et al.* (1995) reported that soybean meal contains about 1 g/kg genistein, which is over 4 x 10^3 times more estrogenic than diethylstilboestrol (a synthetic estrogen).

Soybean has many advantages in poultry feeding as it is a good source of protein with a high concentration of lysine. Also it is a better source of calcium and phosphorus than many cereals (NRC, 1994; McDonald *et al.*, 1995). Furthermore, it is a cheaper source of protein than fishmeal and meat. Soybean not only contains phytoestrogen but a number of stimulatory and inhibitory substances have also been identified in this ingredient. These include allergenic, goitrogenic, anticoagulation and anti-trypsin and chymotrypsin inhibitors which can be inactivated by heating and toasting for simple stomach animals such as laying hens. No reported has been found in regards to inactive of phytoestrogen in soybean during the heat processing. Growth rate, feed efficiency and molecular mechanism in relation to calcium transport have not been elucidated in hens in relation to this constituent.

It is thought that phytoestrogens, as estrogenic compounds, may interact with estrogen receptors in unfilled nuclear estrogen sites in the uterus and possibly in the small intestine (Martin *et al.*, 1978). This interaction could induce the binding of the estrogen receptor with DNA and affect protein synthesis. Such proteins may be associated with calcium transport and calcium retention in pullets potentially increasing the calcium pool before the onset of lay. Although the effect of phytoestrogens on estrogen receptors has been demonstrated in humans by Frank *et al.* (1994), the mechanism is still unknown in laying hens particularly in immature birds during the development of their sexual maturity. In addition non-genomic effect of estrogen receptors may induce the ATPase and consequently could increased calcium absorption in intestine (Picotto et al., 1996). The effect of phytoestrogen may be to compete with estrogen at a estrogen receptor site and thus modulate the activity of estrogen in young birds. Therefore, high level of estrogen at this particular age may induce more calcium absorption by a possible direct effect of estrogen and phytoestrogen on the small intestine as described in chapter 3. This situation may lead a storage of calcium in the bone of young birds, essential for egg production.

In the experiment reported in this chapter, an attempt is made to investigate the role of estrogen and phytoestrogen to switch on the expression of CaBP (calbindin D_{28k}) in the small intestine before onset of lay (Hurwitz, 1964 and 1989). Leghorn pullets (10-14 weeks) were used with 50 μg of E_2 to determine if there was greater expression and potentially more storage of calcium prior to egg laying in regards to phytoestrogen and synthetic estrogen effects.

For these reasons the objective of this chapter will be to focus on the effects of phytoestrogens particularly those in soybean when it is used to replace meat meal in cereal based diets. The interaction between estrogen and soybean (phytoestrogen source) on growth, feed efficiency, mineral (particularly calcium) retention are tested in this experiment. Alkaline phosphatase and ATPase enzyme activities in jejunal tissue, intestinal morphology and calbindin D_{28k} expression with respect to phytoestrogen and exogenous estrogen were also considered.

6.2. Materials and methods

Thirty-two Leghorn pullets with similar initial body weight (796-811 g) at pre-laying stage (10 week old) were chosen. They were reared for 4 weeks until 14 weeks of age (before the onset of lay) in individual cages for the testing the effect of high phytoestrogen diets and synthetic estrogen on feed efficiency and nutrient retention. Rearing conditions were similar to those described in previous chapters. Four treatments combining two levels of estrogen (0 and 50 µg E_2, /kg body weight/day which were administrated by injection every day at 5 pm) and two dietary treatments (meat meal and soybean meal) were used. Soybean replaced meat meal to provide high phytoestrogen in cereal based diets. Treatments were used in a complete random design (2 x 2 x 8) Table 6.1.

Table 6.1: Diets and different levels of estrogen (treatments)

Diets	Estrogen (E_2) µg/kg body weight/day	
1. Meat meal	0	50
2. Soybean meal (phytoestrogen source)	0	50

The growth of oviduct and shell gland, intestinal and gizzard capacity were considered in this study. Feed ingredients in this experiment for the meat meal diet (1) and soybean meal diet (2) using a cereal base diet were prepared as shown below. Both diets were designed to meet the requirements of 10-14 week old Leghorn pullets (NRC, 1994).

Feed formulation for 10-14 weeks Leghorn pullets (Diet composition (g/100 g)

Feed source	**Diet 1**	**Diet 2**
1. Wheat	50.00	50.00
2. Triticale	31.00	30.00
3. Barley	7.00	4.00
4. Meat meal (50% CP)	11.00	–
5. Soybean (48% CP)	–	12.00
6. Lysine (synthetic)	0.15	0.15
7. Methionine (synthetic)	0.25	0.25
8. Rock Phosphate (38% Ca, 17% P)	-	3.00
9. Sunflower oil	0.30	0.30
10. Mineral Vitamins, salt	0.30	0.30
Total	**100.00**	**100.00**
Metabolisable energy (ME)[1]	11.90 MJ/kg	11.80
Crude protein[2]	16.18 %	16.00 %
Calcium[2]	1.26 %	1.23 %
Total phosphorous[2]	0.89%	0.89%
Fibre[2]	2.93 %	3.26%
Fat[2]	2.48%	1.72%
Total phytoestrogen	ND[3]	1.666[4]
Daidzein	ND	0.682[4]
Genistein	ND	0.984[4]

1, Calculated
2, Measured.
3, Non detected (The detected limit for phytoestrogens was 10 ng).
4, Measured, mg/g soybean

Data were subjected in ANOVA and mean values tested by LSD.

6.3. Results

6.3.1. Growth and feed efficiency

The values obtained from growth and feed efficiency measurements are presented in the Table 6.2. Initial body weights were similar (796-811g) for all treatments in this experiment. Although no changes were found in weight gain (g) with different levels of estrogen, soybean significantly reduced (P<0.001) weight gain in comparison with meat meal.

Daily feed intake (g) and feed intake per unit weight (g/100g body weight) were similar between treatments (diets and estrogen levels). Feed conversation ration (FCR) was significantly reduced (P<0.001) for soybean diets (with low efficiency) when compared with meat meal diets. No response was found regarding the effect of estrogen on FCR. The interaction between diets (meat meal and soybean meal) and different levels of estrogen (0 and 50 μg /kg body weight/day) were not significant on growth, feed intake or FCR.

6.3.2. Growth and capacity of the gastrointestinal tract and oviduct

Growth and capacity of the gastrointestinal tract (GIT) per 100 g body weight are shown in the Table 6.3. The growth of the proventriculus and gizzard (PRG) and also their capacity were not significantly different between diets or levels of estrogen. Although it appeared that the soybean meal had greater values for the most measurements, the growth and capacity of PRG declined with the high estrogen dose (50 μg). The growth and capacity of Intestine were significantly greater (P<0.01) with soybean meal than meat meal. No significant differences were observed in the

growth and capacity of intestine with respect to estrogen effect, while it seems some reduction in this aspect by 50 μg of E_2.

No significant differences were identified in oviduct and shell gland growth between diets or the level of estrogen. Although the growth of these organs increased slightly with 50 μg of estrogen and meat meal. The interaction between soybean and estrogen (soybean x E_2 50) was significant ($P<0.05$) reduction in the growth of the proventriculus and gizzard (PRG) compared with the other treatments. No significant differences were identified by the interaction between estrogen and either phytoestrogen content in soybean or meat meal diets on growth and capacity of gastrointestinal tract as well as in oviduct and shell gland weight (Table 6.3).

Table 6.2: Feed intake and growth in 10-14 week old Leghorn pullets in, response to, different diets or treated with different levels of estrogen.

	Diets		μg E_2/ kg body wt/day		
	Meat meal	**Soybean**	**0**	**50**	**SEM**
A. Growth					
Initial body weight (IBW, g)	796.00	811.00	805.00	802.00	17.40
Weight gain (WG, g)	449.00[a]	310.00[b]	348.00	412.00	33.20
B. Feed					
Daily feed intake (DFI, g)	56.80	55.50	54.80	57.50	2.41
Feed intake (FI, g/100g wt)	241.00	232.60	231.40	242.10	11.60
Feed conversation ratio (FCR, feed ,g / WG, g)	4.43[b]	5.40[a]	5.02	4.81	0.13
C. Source of variation	**IBW**	**WG**	**DFI**	**FI**	**FCR**
Diets	NS	****	NS	NS	****
E_2	NS	NS	NS	NS	NS
Diets x E_2	NS	NS	NS	NS	NS

Means values on the same row without a common superscript are significantly different****,($P<0.001$)
E_2, estrogen; SEM, Standard error of means; NS, Not significant.

Table 6.3: Gastrointestinal tract weight, capacity and oviduct weight (g/100g body wt.) in response to different diets or treated with different levels of estrogen in 14 week old Leghorn pullets.

	diets		μg E_2/ kg body weight/day			
	Meat meal	**Soybean**	**0**	**50**	**SEM[1]**	
A. Growth						
Prov./gizzard wt. (PRG)[2]	3.59	3.90	3.84	3.65	0.12	
Capacity (PRG C)[3]	0.748	0.845	0.858	0.735	0.07	
Small intestine weight (SI)[4]	3.08[b]	4.01[a]	3.66	3.43	0.31	
Capacity (SIC)[5]	0.430[b]	0.973[a]	0.750	0.653	0.14	
Oviduct weight (OW)	0.66	0.38	0.48	0.56	0.14	
Shell gland weight (SGW)	0.285	0.141	0.210	0.216	0.060	
B. Source of variation	**PRG**	**PRGC**	**SI**	**SIC**	**OW**	**SGW**
Diets	NS[7]	NS	**	**	NS	NS
E_2[6]	NS	NS	NS	NS	NS	NS
Diets x E_2	*	NS	NS	NS	NS	NS

Means values on the same row without a common superscript are significantly different, * , (P<0.05**, (P<0.01); , NS, not significantly; 1, SEM, Standard error of means; 2, PRG, proventriculus and gizzard weight; 3, PRGC, proventriculus and gizzard capacity; 4, SI, small intestine weight; 5, SIC, small intestine capacity; 6, E_2, Estrogen; NS, not significant.

6.3.3. Phytoestrogen intake and plasma phytoestrogen levels

The intake of phytoestrogen and their plasma concentration are presented in Table 6.4 and Figure 6.1, respectively. No differences were detected with respect to the 0 and 50 µg E_2/kg/body weight/day on soybean intake, g; total phytoestrogen, TPE; daidzein, D and genistein intake, G; by those birds fed with soybean diet (Table 6.4). Phytoestrogens were not detected in cereals (wheat, barley, triticale) and also meat meal, although some phytoestrogens were detected in the plasma of those birds that consumed non-estrogenic diets (wheat, barley, triticale and meat meal) (Figure 6.1). The concentration of TPE, D and G in the plasma of 14 week old Leghorn pullets maintain on the soybean diet was dramatically greater ($P<0.001$, $P<0.005$ and $P<0.001$ respectively) about 5 times, when compared with birds fed the meat meal diet.

No significant difference was indicated between interaction of estrogen levels (0 and 50 µg) and diets (soybean and meat meal) on plasma concentration of phytoestrogen.

Table 6.4: Soybean and phytoestrogens intake in 10-14 week old Leghorn pullets

Intake	µg E_2 /kg body weight /day		SEM
	0	50	
Soybean meal (g/day)	6.02	6.33	0.33
Total phytoestrogen(TPE, mg/day)	10.03	10.56	0.54
Daidzein (D, mg/ day)	4.11	4.33	0.22
Genistein (G, mg/ day)	5.92	6.23	0.32

E_2, Estrogen; SEM, Standard error of mean.

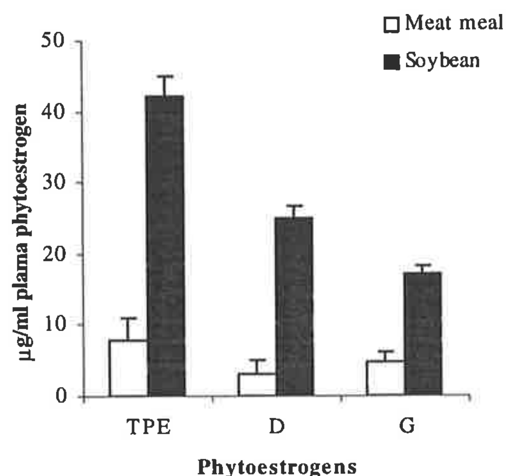


Figure 6.1: Plasma concentration of phytoestrogens in maintained on either meat meal or soybean diets. Total phytoestrogen (TPE), diadzein (D), genistein (G). columns with different colors within the concentration of phytoestrogens (TPE (P<0.001), D (P<0.005) and G (P<0.001) differ significantly.

6.3.4. Nutrients retention

The effect of exogenous estrogen diets (meat meal or soybean meal) on nutrient retention are presented in Table. 6.5. Dry matter retention was significantly increased (P<0.01) by estrogen (50 µg). Although no significant response was found on dry matter retention by diets, it seems that the effects of soybean meal were generally greater than those from meat meal. Crude protein retention was increased significantly (P<0.001) with soybean meal compared with meat meal. Also there was an increase in CP retention with estrogen (50 µg) compared with nil estrogen. Calcium retention was increased significantly (P<0.001) and (P<0.005) by soybean diets and estrogen dose (50 µg) respectively, although the increase was greater with soybean. There were significantly greater effects with soybean in phosphorous (P<0.001), sodium (P<0.001) and magnesium (P<0.005) retention compared with meat meal. The reverse effect was found in potassium retention. No significant

differences were found in regard to the estrogen dose (50 μg) on phosphorous, magnesium and potassium retention, although a large effect was identified with 50 μg E_2 on these items. Sodium was significantly reduced (p<0.001) by this dose of estrogen.

The interaction between soybean and estrogen (0 and 50 μg) on calcium retention was significantly greater (P<0.001) than interaction between other treatment groups. A similar significant (p<0.005) interaction was found on sodium retention.

Table 6.5: The retention of various nutrients by 10-14 week old Leghorn pullets fed different diets or treated with different levels of estrogen.

	diets		μg E_2/ kg body wt/day		
	Meat meal	**Soybean**	**0**	**50**	**SEM**
A. Nutrients retention (%)					
Dry matter (DM)	71.8	74.8	69^{b}	77.6^{a}	2.12
Crude protein (CP)	31.6^{b}	50.1^{a}	37.4	44.3	3.7
Calcium (Ca)	37.9^{b}	87.2^{a}	56.8^{b}	68.3^{a}	2.23
Phosphorous (P)	22.1^{b}	60.1^{a}	37.5	44.7	3.80
Magnesium (MG)	15.5^{b}	37.9^{a}	24.6	28.8	4.46
Sodium[2] (Na)	27.6^{b}	86.8^{a}	61.8^{a}	52.6^{b}	1.87
Potassium (K)	88.4^{a}	26.4^{b}	53.4	61.4	3.49

B. Source of variation	**DM**	**CP**	**Ca**	**P**	**Mg**	**Na**	**K**
Diets	NS	***	****	****	***	****	****
E_2	**	NS	***	NS	NS	***	NS
Diets x E_2	NS	NS	****	NS	NS	***	NS

Means values on the same row without a common superscript are significantly different, * ,(P<0.05), ** , (P<0.01), *** ,(P<0.005), **** ,(P<0.001); SEM, Standard error of means; E_2, Estrogen

6.3.5. Intestinal mucosal morphology

The morphology of villi in different regions of the intestine are shown in the Table

6.6. No significant differences were observed in the crypt depth at the duodenum jejunum or ileum with respect to diet or estrogen level with the exception of duodenal crypt depth which was significantly reduced ($P<0.005$) by estrogen (50 μg/ kg body weight/day). Villus height was dramatically reduced ($P<0.005$) in the duodenum by soybean meal when compared with the meat meal, although there was no significant differences in villus heights in jejunal and ileal tissue in response to either diet or estrogen treatment.

Villus surface area at the duodenum was reduced markedly ($P<0.001$) by soybean meal when compared with meat meal. No significant changes were found in the surface area of other regions due to diet or estrogen level.

Villi heights decreased from the duodenum to ileum. No significant effect was observed the interaction between diet (meat meal and soybean meal) and different levels of estrogen (0 and 50 μg) in the different regions of the intestine.

Table 6.6: Intestinal mucosal morphometry in 14 week old Leghorn pullets fed different diets or treated with different levels of estrogen.

	diets			E2/μg kg body weight/day					
	Meat meal	Soybean		0		50		SEM	
A. Cypt depth (VR, μm)									
Duodenum (D)	318.60	343.40		357.20[a]		304.80[b]		11.37	
Jejunum (J)	279.90	292.50		280.80		291.60		11.41	
Ileum (L)	209.00	201.00		196.50		214.00		6.33	
B. Villus height (VH, μm)									
Duodenum	2418.00[a]	1994.00[b]		2185.00		2227.00		78.70	
Jejunum	1796.00	1701.00		1724.00		1772.00		75.40	
Ileum	1134.00	1189.00		1099.00		1224.00		55.90	
C. Villus surface area (VSF, mm^2)									
Duodenum	0.590[a]	0.440[b]		0.514		0.516		0.026	
Jejunum	0.387	0.339		0.375		0.350		0.021	
Ileum	0.233	0.235		0.219		0.249		0.013	
D. Source of variation	DCR	JCR	LCR	DVH	JVH	LVH	DVSF	JVSF	LVSF
Diet	NS	NS	NS	***	NS	NS	****	NS	NS
E_2	***	NS	NS	NS	NS	NS	NS	NS	NS
Diets x E_2	NS	NS	NS	NS	NS	NS	NS	NS	NS

Mean Values on the same row without a common superscript are significantly different; NS, not significant; E2, estrogen, SEM, standard error of means. * , (P<0.05), **(P<0.01), *** , (P<0.005), **** , (P<0.001)

6.3.6. Enzyme activity

Enzyme activities were assessed in homogenates of jejunum tissue. Alkaline phosphatase and ATPase enzyme activities were present and the values obtained are shown in the Figure 6.2 (A-D). Fig. 6.2 (A) shows alkaline phosphatase was significantly ($P<0.001$) greater in soybean meal than meat meal. All ATPase enzyme activity including Ca and Mg ($P<0.001$) and Ca/Mg ($P<0.005$) were significantly reduced by the soybean diet when compared with those from the meat meal diet with the exception of Na/K ATPase activity where there was no significant reduction (Fig. 6.2, B)

Although, there was a small increase due to estrogen (50 μg) effect in all enzyme activities, no significant effect was observed by estrogen level (0 and 50 μg) on alkaline phosphatase and ATPase activity (Fig. 6.2, C and D) respectively. There were no significant interactions between diets (meat meal and soybean) or estrogen level (0 and 50 μg) on alkaline phosphatase and ATPase activity.

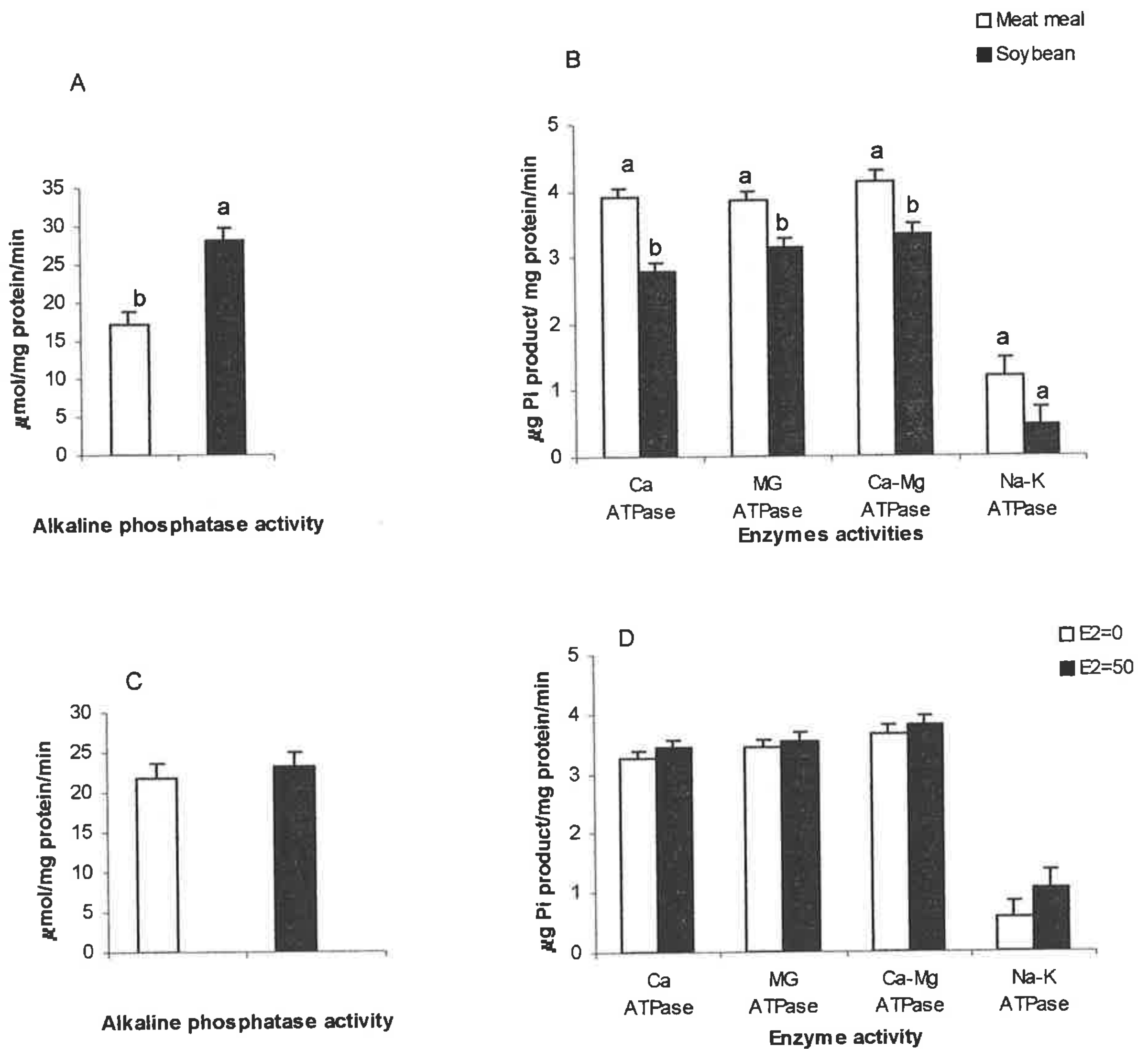
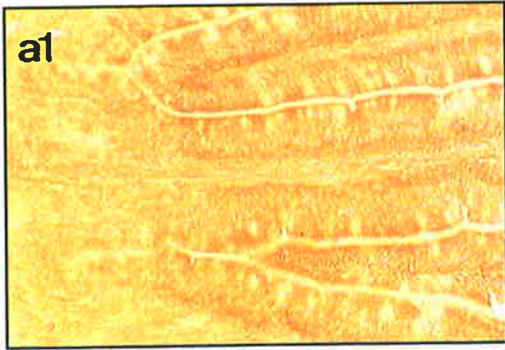
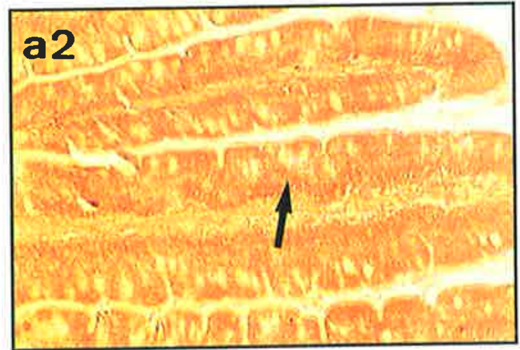
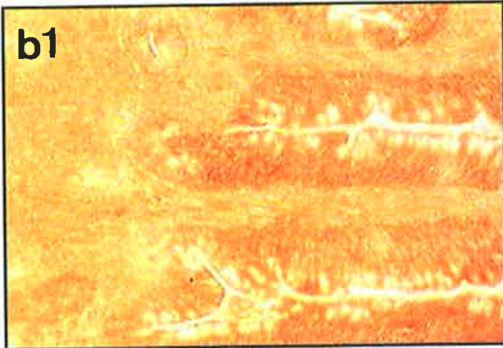
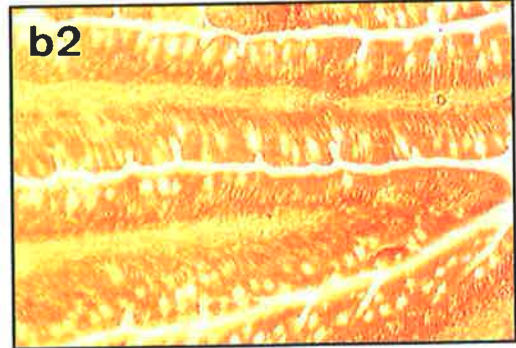
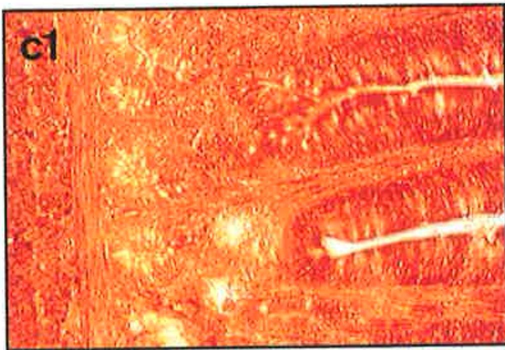
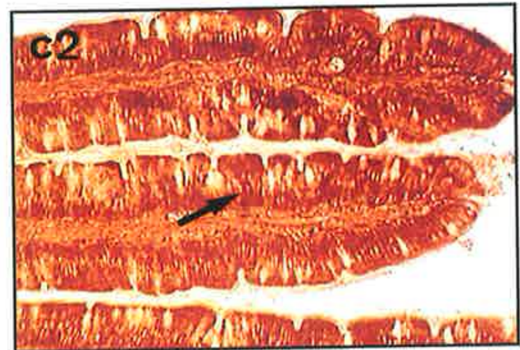
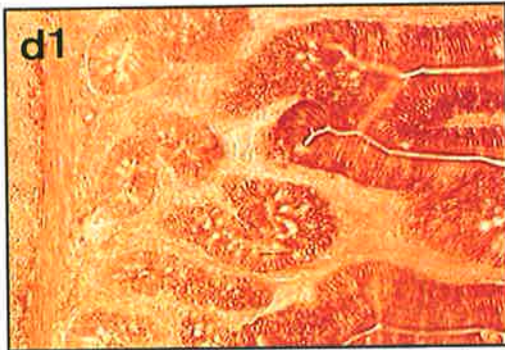
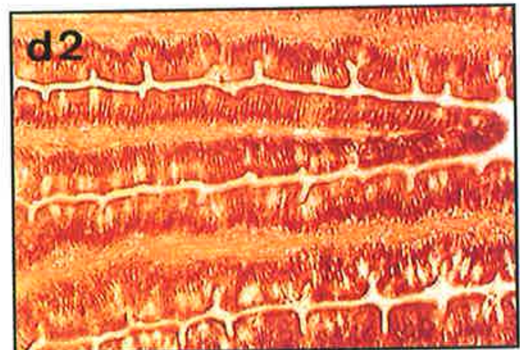


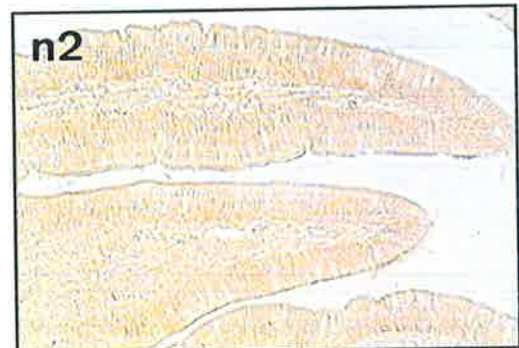
Figure 6.2: Alkaline phosphatase (A) and ATPase enzyme (B), activities in response to meat meal and soybean meal diets, respectively; Alkaline phosphatase (C) and ATPase enzyme (D), activities in response to different levels of estrogen (E_2, µg/ kg body weight/day) respectively. For diet Columns with different letters within the enzyme activity differ significantly for alkaline phosphatase, Ca and Mg ATPase ($P<0.001$) and Ca/Mg ATPase ($P<0.005$). not significant for estrogen levels (0 and 50µg)

6.3.7. Expression of calcium binding protein (calbindin D_{28k})

The expression of calcium binding protein in the jejunum tissue in regard to diet (meat meal and soybean meal) and level of estrogen are shown in Plate 6.1. Jejunal Enterocytes expression of CaBP (calbindin D_{28k}) was greater in soybean meal compared with meat meal diets, regardless to estrogen dose. The expression of calbindin D_{28k} was localised in the entrocytes absorptive cell of epithelial cell along of villus length.

Plate 6.1: Immunohistochemical localisation of calbindin D_{28K} in crypt (1) and villus (2) regions of typical jejunal tissue taken from 14 week old Leghorn pullets in response to cereal and soybean meal diets and different levels of exogenous estrogen. Panels a and b represent tissue taken from hens maintained on cereal diet and treated with 0 or 50 µg estrogen/kg body weight/day, respectively. The effect of a soybean meal diet for the same estrogen treatments is represented in panels c and d. Typical negative controls are represented in panels labelled with n. Arrows indicate typical positive stain in epithelial cells


a1

a2

b1

b2

c1

c2

d1

d2

n1

n2

6.4. Discussion

6.4.1. Growth and feed efficiency

The significant reduction ($P<0.001$) in weight gain by pullets fed soybean meal compared with meat meal may have been due to the amount the phytoestrogen in soybean meal (absent in meat meal). The depression of feed intake and also the reduction in efficiency (FCR) with soybean meal diets compared with meat meal diets was illustrated in a reduced weight gain. This was also reported by Austic and Nesheim (1990) and Hunton (1995).

Measurements of daily feed intake, feed intake (in g and g /100 g body weight) and FCR were made in the present experiment. No significant differences were found in daily feed intake and feed intake in regard to diet or estrogen level. This is in agreement with the results of Adams *et al.* (1950) and Robey *et al.* (1988). Feed conversion (increase FCR value) was significantly increased by soybean meal. The decrease in conversion efficiency by soybean meal compared with meat meal may be due to phytoestrogens present in soybean, this has not been reported elsewhere. However other component such as phytic acid which binds with proteins as an inhibitor (Sebastian *et al.,* 1998), or lectins which are found in high proportion in soybeans, 30-40% of natural lectins remain active after heat processing (Fuller, 1991), these may affect the nutrient absorption. The main reason for adverse effect of lectins is high resistance of this antinutritive to proteolytic enzyme breakdown in the intestine (Fuller, 1991). It seems that feed conversion may be worse in pre-laying hens than when hens are laying, as their tissues prepare for egg production. Pullets, consume a large quantity of feed but it is not matched by weight gain which leads to

an increase in FCR (less efficient use). As a result of this they may be under weight at this stage as a noted by Hunton (1995). Similar results have been obtained in the present experiment.

6.4.2. Growth of the gastrointestinal tract and oviduct

The results obtained in this experiment indicate that overall, gastrointestinal tract growth and its capacity were higher with the soybean diet and estrogen (50 μg) except for proventriculus and gizzard in Leghorn pullets. This may be due to plant cell wall of soybean diet in comparison with meat meal although these diets have been balanced in protein, energy, amino acids, minerals and vitamins, but physical feature of soybean meal with particular fibre content may induce growth and therefore and its capacity as described by (Yu and Chiou, 1996)) in rabbit. Therefore the physical proportion of nutrients in soybean may promote development of the small intestine. The oviduct and shell gland weight were reduced to a degree by soybean, although, it appeared this reduction was not significant and has not been shown by others. This could be related to the high level of phytoestrogen of soybean as an inhibiting factor on the reproduction system (Lien *et al.*, 1985).

The growth and capacity of gastrointestinal tract decreased to a degree with synthetic estrogen, although the difference was not significant. The reason for this could be related to the adverse effect of estrogen on intestinal mucosa morphometry. This adverse effect might be due to the effect of chemical composition in 17β-estradiol. Table 6.3 indicated oviduct and shell gland weight was increased by high level (50 μg) estrogen but not significantly. This pattern was also shown by Qin and Klandorf (1995).

6.4.3. Phytoestrogen intake and plasma phytoestrogen levels

The daidzein (D) and genistein (G) were identified in this experiment as the main phytoestrogens sources in soybean diets. This has been shown previously by Setchell and Welsh (1987) and Wang, *et al.* (1990). Total phytoestrogens were calculated as the sum of these two phytoestrogens. They were not detected in the other components of the cereal base diets e.g. wheat, barley and triticale (Franke *et al.*, 1994). Also they were not detected in meat meal.

The results of the present experiment demonstrated that there were no significant differences in TPE, D and G daily intake in soybean diets (mg/g soybean/day) under the influences of different levels of synthetic estrogen (0 and 50 μg). Plasma phytoestrogen concentration (TPE, D and G) were significantly higher in the birds fed soybean meal compared with those groups fed meat meal. Although no phytoestrogens were detected in meat meal or the other ingredients (wheat, barley and triticale), phytoestrogens were detected in plasma of those hens fed these ingredients. This could be related to the presence of phytoestrogens precursors that are not detectable until metabolised by the animal (Personal communication, Dalais, 1998) This sort of accumulation of phytoestrogen has been observed in plasma of wild birds exposed to low level of phytoestrogen in their diets (Lien *et al.*, 1985; Lien and Cain, 1987).

6.4.4. Nutrient retention

The results reported in Table 6.5 showed that no significant differences occurred between the meat meal and soybean diets with regard to dry matter retention,

although there was significant increase with estrogen dosage (50μg). In this latter situation, significant differences could be due to greater activity of the oviduct and shell gland in preparation for egg production. As described in the results of growth and efficiency, in this chapter oviduct and shell gland weight were significantly increased by high dose estrogen. This suggests that additional energy and nutrients are required and cause an increase in feed intake. In this respect nutrient retention of most ingredients (CP, Ca, P, Mg and K) was increased by 50 μg of E_2. The effect was significantly greater ($P<0.001$) in regard to calcium retention. This may be related to the positive effect of estrogen on calcium transport in the small intestine which was demonstrated by Arjmandi *et al.* (1993) in humans. Sodium retention however was reduced significantly ($P<0.001$) by 50 μg of estrogen dose.

The retention of nutrients (e.g CP, Ca, P, Mg and Na) was significant effected by high phytoestrogens diets (soybean in comparison to the meat meal diet). The same effects were found on nutrient retention by estrogen. Franke *et al.* (1994) suggested that the phytoestrogen may have the same effect as estradiol on protein synthesis which is associated with calcium transport in plasma cell membranes in human. The effect of soybean meal to increase calcium retention may related to high protein retention, because this can be associated with high calcium retention (Clarence *et al.*, 1995).

In the present case, it is clear a large proportion ($P<0.001$) of potassium retention occurred with meat meal compared with the soybean. This may have been due to potassium in meat meal and the availability of that compared with potassium in the soybean meal. No other reason has been identified in this aspect. The significant interaction $P<0.001$) of soybean diets and high level of E_2 (50 μg) on calcium

retention demonstrated a possible association between estrogen, phytoestrogen and estrogen receptor in the small intestine for calcium transport in Leghorn pullets in this experiment. As Huwitz, (1964 and 1989) and Hunton (1995) demonstrated, prior to the onset of the egg production, calcium retention is greatly increased. Increased Na retention may effect calcium retention as a co-carrier in this process (Van Os, 1987)

6.4.5. Intestinal mucosal morphology

Estrogen treatment and diet, can have a considerable influence on intestinal structure and mucosal morphology by affecting the ability of nutrients to be absorbed particularly in regard to the high demand of calcium in pre-lay hens. The data of the present experiment showed that there was no response to the meat meal and soybean diets, or the level of estrogen (0 and 50 µg) on the development of crypt depth at different regions (duodenum, jejunum and ileum) of the small intestine. The high level of estrogen (50 µg) significantly decreased duodenum crypt depth. The increased height and surface area of villi could increase the potential of the small intestine to digest and absorb nutrients, in particularly calcium (Smith and Peacock, 1989; Alberts *et al.* 1994).

Duodenal villus height ($P<0.005$) and villus surface area ($P<0.001$) were significantly reduced by the phytoestrogen diet (soybean meal) compared with meat meal. This reaction might be due to the nature of soybean meal as described in this chapter and also to high level of phytoestrogen that to effect of different regions of the villus. This has not been identified in literature by others.

Overall exogenous estrogen (and high phytoestrogen diets) reduced duodenal villus height and surface area. This action may reduce the ability of the small intestine to digest and absorb materials and consequently may change calcium absorption in pre-laying Leghorn pullets. In contrast to the point, increased calcium retention by hens on soybean meal, may be due to increase protein retention Civitelli and Avioli (1994). In addition no significant differences were found in jejunal morphometry with respect to both diets (soybean and meat meal). Although no significant differences were observed in villus height and surface area by 17β-estradiol, the increased calcium retention may be related to the small increase in duodenal and jejunal villus height and also duodenum surface area by 50 μg of E_2 (Table.6.6).

In addition the effect of 50 μg of E_2 on duodenum and jejunum villus site as the main parts of small intestine for calcium absorption Hurwitz *et al.* (1972) and Hurwitz, (1989), other factors could be involved in this pattern. These factors includes: high activity of alkaline phosphatase in the jejunal tissue of those hens which were fed soybean meal (Van Os, 1987), biochemical reaction for the storage and movement of Ca^{2+} intracellular (Bronner, 992) as described in chapter 5, basolateral membrane calcium transport and also high demand for calcium to increase the calcium pool in the bone at this particular age (Gilbert, 1983; Hurwitz, 1989).

6.4.6. Intestinal enzyme activity

Fig. 2 (a) and 2 (b) illustrate alkaline phosphatase and ATPase enzyme activity respectively. Alkaline phosphatase activity was significantly greater ($P<0.001$) in jejunal tissue by soybean meal than meat meal. This could be one of the reason for increase calcium retention by soybean meal (Van Os, 1987). A significant reduction

(P<0.005) were recorded in Ca, Mg and Ca/Mg ATPase activity with no change in Na/K ATPase activity in jejunal tissue by the soybean meal compared with the meat meal diet. This may be due to some components in the soybean meal such as phytic acid and particularly lectins as protein inhibitors which could affect the enzyme activity (Fuller, 1991; Sebastian *et al.*, 1998)

Although alkaline phosphatase and ATPase enzyme activity were not significantly different due to the estrogen level, a small increase of the enzymes activities were observed by a high level of estrogen (50 μg). A similar result has been reported by Qin and Klandorf, (1993) and Picotto *et al.* (1996).

6.4.7. Expression of calcium binding protein in the jejunal tissue

No differences were identified in expression of calbindin D_{28k} at jejunal tissues with respect to exogenous estrogen or phytoestrogen (soybean diet) or with their interaction. Although the expression of calbindin D_{28k} in the small intestine has been demonstrated by numerous studies (Jande *et al.*, 1981; Bar *et al.*, 1990), the effect of phytoestrogen an its expression has not been elucidated.

In contrast to the higher retention of calcium by soybean meal compared with meat meal diet, no differences were recognised in expression of calbindin D_{28k} due to treatments. However, this high calcium retention may be concerned to the other reaction which are described in this chapter (e.g. high activity of alkaline phosphatase and greater protein retention).

6.4.8. Conclusion

The results of this experiment showed that similar effects on feed intake occurred with either meat meal or soybean meal or with different levels of estrogen effect (0 and 50 μg). Weight gain and feed conversion however, were significantly decreased by soybean meal with a high proportion of phytoestrogen (daidzein and genistein). There was no significant effect of estrogen level (0 and 50 μg) on soybean meal and consequently on phytoestrogens (daizein and genistein) intake, but dramatic and significant differences were observed in the plasma concentration of phytoestrogens (daizein and genistein) in hens fed soybean meal compared with a meat meal diet. The presence phytoestrogens in the plasma of those groups of hens fed with meat meal could be due to the presence of phytoestrogen precursors in the meat meal diet only detectable once metabolised by the animal (F. Dalais, personal communication, 1998).

The high estrogen dose (50 μg) caused a small increase (but not significant) in alkaline phosphatase and ATPase in particular calcium ATPase activity. In contrast the activity of these enzymes were reduced by soybean meal with exception of alkaline phosphatase. No predominant changes were recorded in intestinal mucosal structure with the exception of duodenum villus surface area and height with plant estrogenic treatments (soybean). The expression of calbindin D_{28k} in the tissue of the small intestine in this particular age of hens regarding to phytoestrogen was greater than meat meal diet this was correspond with high calcium retention by soybean meal.

Nutrient retention, particularly calcium retention, and also alkaline phosphatase

activity increased remarkably with dietary phytoestrogens (soybean meal) and a high level (50 μg) of synthetic estrogen. This demonstrated that pre-laying Leghorn pullets at this age (14 week old) attempt to increase the calcium pool for their maintenance and also prepare their body for egg and egg shell production in the next stage (onset of lay). These results suggest that the basis for association of phytoestrogen on calcium retention and consequently in egg production and shell quality remains unclear, at this stage of development.

CHAPTER SEVEN

INTESTINAL FUNCTION AND REPRODUCTIVE CAPACITY OF 18-22 WEEK OLD LEGHORN PULLETS IN RESPONSE TO EXOGENOUS ESTROGEN AND HIGH PHYTOESTROGEN DIET

"Phytoestrogen could be a factor influencing reproduction in wild quail population"
(Lien et al, 1987)

7.1. Introduction

Some plants contain components that have weak estrogenic activity (phytoestrogens). Their existence in soybean has been recognized by several investigators (Kudou *et al.*, 1991 and Coward *et al.*, 1993). Phytoestrogens and their metabolites have been identified also in the human biological fluids. In particular, a component which is in soybean has been shown to be excreted in the urine of man (Baghurst, 1997). Although much of our understanding of phytoestrogens stems from research conducted on clover disease in farm animal over fifty years ago (Bennetts *et al.*, 1946) as emphasized by Baghurst (1997), little is known with respect to their effect on poultry reproductive function and their action in pre-laying hens during the development of their reproduction system, and consequently, egg production and egg shell formation. Lien and Cain (1985) reported that consuming feed containing more than 1 mg/day of phytoestrogen may impair the reproduction of 40 week old laying hens.

Soybean is frequently a major component of poultry diets, as a source of protein. After extraction of oil, soybean seed, as a protein meal, contains up to 50 percent protein and is an excellent source of this nutrient for poultry. The protein present is a good source of all the essential amino acids and with a suitable source of energy and minerals is of great benefit to the poultry industry (Evan, 1985; Mcdonald *et al.*, 1995).

On the other hand, soybean, because of its high concentration of phytoestrogens may have an adverse effect on sexual maturation and on the onset of lay in laying hens. It is known that the major metabolic pathway of these estrogens involves binding to the estrogen receptors in the nucleus of cells (Beato, 1989). Transcriptional activity may be changed by binding of the estrogen receptors to estrogen- receptor-element (ERE) and this procedure could induce the synthesis of a particular protein which is essential to calcium transport. This knowledge has led to the recent recognition that phytoestogens as plant compounds may regulate gene expression, mediated by ERE similar to the binding of 17β-estradiol by estrogen receptors in human cells (Arjmandi *et al.,* 1993; Baghurst, 1997). Therefore, phytoestrogens as estrogenic components may induce or block protein synthesis, which may be associated with calcium transport. Non-genomic reactions of estrogen receptor could be another option in this case.(Picotto *et al,* 1996) Thus, understanding the actual role of the major phytoestrogens including those in soybean (daidzein and genistein) and their effect on egg production and shell formation is of priority and needs to be clarified for the poultry industry.

As the pervious chapter considered the function of phytoestogen in the 10-14 week old Leghorn pullets before onset of egg production. This chapter covers work which investigated the effects of phytoestrogen when soybean was used as a replacement for meat meal in cereal based diets in 18-22 weeks Leghorn pullets. Therefore, effects of synthetic estrogen (17β-estradiol) or soybean (phytoestrogen) on growth, feed intake, feed conversion and mineral retention, (especially calcium retention) during shell formation are considered. To determine these affects, it was necessary to examine alkaline phosphatase and ATPase enzyme activity in jejunal tissue, intestinal morphology as well as the expression of calcium binding protein.

7.2. Materials and methods

Twenty-eight (18-week-old) Leghorn pullets were used in this experiment. They were housed for four weeks in individual cages. Lighting regime and all rearing conditions were similar to those in previous experiments. A daily intramuscularly injection of 0 or 50 μg estrogen /kg body weight/day dissolved in the peanut oil was used as a source of exogenous estrogen. The estrogen was injected every afternoon at 5 pm. A further treatment used soybean meal as a replacement for meat meal as a source of phytoestrogen in diets based on cereal. Therefore, four treatments were arranged in a complete random design (2 x 2 x 7) in this experiment (Table 7.1).

Table 7.1: Diets and different levels of estrogen (treatments)

Diets	Estrogen (E_2) μg/kg body weight/day	
1. Meat meal	0	50
2. Soybean meal (phytoestrogens source)	0	50

Nutrient retention feed efficiency, growth and capacity of the gastrointestinal tract, liver, oviduct and shell glands were analysed in this part of the study. Interactions between diets (soybean meal and meat meal) and synthetic estrogen (0 and 50 μg) were tested. Feed ingredients in this experiment meat meal (diet 1) or soybean meal (diet 2) were used based on the recommendation of NRC (1994) for 18-22 week old Leghorn pullets. There were compared as follows:

Feed formulation for 18-22 week-old Leghorn pullets (diet composition g/100g)

Feed sources	Diet 1	Diet 2
1. Wheat	50.00	50.00
2. Triticale	21.00	22.00
3. Barley	8.00	2.00
4. Meat meal (50% CP)	15.00	5.00
5. Soybean (48% CP)	-	12.00
6. Lysine (synthetic)	0.15	0.15
7. Methionine (synthetic)	0.25	0.25
8. Rock phosphate (38.5% Ca, 17% P)	5.00	8.00
9. Sunflower oil	0.30	0.30
10. Minerals, Vitamins, Yolk colorant	0.30	0.30
Total	**100.00**	**100.00**
Metabolizable energy (ME)[1]	11.55 MJ/kg	11.40
Crude protein[2]	17.09 %	17.30 %
Calcium[2]	3.52 %	3.65 %
Total phosphorous[2]	1.95 %	2.02 %
Fibre[2]	2.74 %	2.93%
Fat[2]	2.65%	1.99%
Total phytoestrogen	ND[3]	1.666[4]
Daidzein	ND	0.682[4]
Genistein	ND	0.984[4]

1, Calculated

2, Measured.

3, Non detected (detected limit in analytical method was 10 ng

4, Measured, mg/g soybean

Data was subjected to ANOVA (analysis of variance) and the resulting mean values compared using LSD.

7.2.1. Phytoestrogen identification and measurement

The phytoestrogens, daidzein and genistein were isolated by high performance liquid chromatography (HPLC) as described in chapter 2 based on the methods of (Reinli and Block, 1996).

7.3. Results

7.3.1. Growth and feed efficiency

Growth and feed efficiency are shown in the Table 7.2. No significant differences were present in initial body weight (1.37-1.38, kg) for diets (meat meal and soybean meal) and estrogen levels (0 and 50μg /kg body weight /day). There was no significant difference in weight gain.

The results from Table 7.2 indicated that there were no significant effects between treatments on daily feed intake (DFI, g), feed intake per unit weight (FI, g/100g body weight) and feed conversion ratio (FCR). Also no significant effects were obtained in growth and feed efficiency overall from the interaction between diets (meat meal and soybean meal) and estrogen levels (0 and 50 μg).

7.3.2. Growth of gastrointestinal tract and oviduct

Gastrointestinal growth and also oviduct and liver weight are shown in the Table 7.3. The growth of proventriculus and gizzard (PRG) and their capacity were greater in birds maintained on a soybean meal compared with meat meal. The increase was significantly different ($P<0.05$) in PRG capacity.

The small intestine, oviduct and liver weight were decreased by the soybean meal diet. In contrast, it seems the capacity of small intestine and shell gland weight were increased with this component. Although liver weight increased with the level of estrogen (50 μg), the other parameters were reduced by the high level of estrogen

compared with low level (0 μg). The differences, however, were not significant. No significant effects were found between interaction of diets (meat meal and soybean meal) and different level of estrogen (0 and 50 μg) on growth and capacity of gastrointestinal tract as well as oviduct and liver weight.

Table 7.2: Feed intake and growth, in response to different diets or treated with different levels of estrogen in 18-22 week old Leghorn pullets

Feed intake and growth	Diets		μg E_2/ kg body weight/day		
	Meat meal	**Soybean**	**0**	**50**	**SEM**
A. Growth					
Initial body weight (IBW, Kg)	1.37	1.38	1.38	1.38	0.04
Weight gain (WG, g)	165.00	219.00	216.00	168.00	48.30
B. Feed					
Daily feed intake (DFI, g)	79.20	83.50	86.70	76.00	5.52
Feed intake (FI, g/100g body)	155.20	158.30	163.90	149.90	11.25
Feed conversion ratio (FCR, egg ,g /feed, g)	2.05	2.10	2.12	2.04	0.12
C. Source of variation	**IBW**	**WG**	**DFI**	**FI**	**FCR**
Diets	NS	NS	NS	NS	NS
E_2	NS	NS	NS	NS	NS
Diets * E_2	NS	NS	NS	NS	NS

E_2, estrogen; SEM, Standard error of means; NS, no significantly

Table 7.3: Gastrointestinal tract weight, capacity and oviduct weight (g/100g body wt.) in response to different diets or different levels of estrogen in 22 week old Leghorn pullets.

	Diets		µg E_2/ kg body weight/day		
	Meat meal	Soybean	0	50	SEM
A. Growth					
Prov./gizzard wt. (PRG[1])	3.62	3.88	3.82	3.67	0.11
Capacity (PRGC[2])	1.01^{b}	1.26^{a}	1.22	1.05	0.08
Small intestine weight (SIW,[3])	3.66	3.60	3.73	3.52	0.18
Capacity (SIC[4])	0.960	1.03	1.05	0.934	0.12
Oviduct weight (OW,)	2.76	2.72	2.78	2.69	0.20
Shell gland weight (SGW,)	0.852	0.914	0.908	0.858	0.06
Liver weight (LW,)	2.43	2.34	2.19	2.59	0.17

B. Source of variation	**PRG**	**PRGC**	**SIW**	**SIC**	**OW**	**SGW**	**LW**
Diets	NS	*	NS	NS	NS	NS	NS
E_2	NS	NS	NS	NS	NS	NS	NS
Diets x E_2	NS	NS	NS	NS	NS	NS	NS

Mean values on the same row without a common superscript are significantly different (*, $P<0.05$); 1, proventriculus and gizzard growth (PRG); 2, The capacity of proventriculus and gizzard (PRGC); 3, small intestine weight (SIW); 4, small intestine capacity (SIC); E_2 , estrogen; SEM, standard error of mean.

7.3.3. Phytoestrogen intake and plasma phytoestrogen levels

Phytoestrogen intake and the plasma concentration of phytoestrogen are presented in the Table 7.4 and Fig. 7.1 respectively. The level of phytoestrogen in the soybean diet was extremely high, but phytoestrogen was not detected in meat meal and the cereal based diet. No differences were observed in intake of (soybean meal, g; total phytoestrogens, TPE; daidzien, D and genistein, G; mg/ day) due to 0 and 50 μg of E_2 treatments. Significant effects were identified in the plasma phytoestrogens (TPE, $P<0.001$; D, $P<0.005$; G, $P<0.001$) in those groups of hens which fed with soybean compared with meat meal diets (about 7-10 times greater). In contrast to the non-detection of phytoestrogen in meat meal and the cereal diet, some phytoestrogens were identified in plasma of those hens which consumed the meat meal diet.

Table 7.4: *Soybean and phytoestrogens intake in 14 week old Leghorn pullets

	μg E_2 /kg body weight /day		
Intake	**0**	**50**	**SEM**
Soybean meal (g/day)	10.37	9.67	0.63
Total phytoestrogen (TPE, mg/ day)	17.28	16.12	1.05
Daidzein (D, mg/ day)	7.07	6.59	0.43
Genistein (G, mg/day)	10.20	9.51	0.62

E_2, Estrogen; SEM, Standard error of mean.

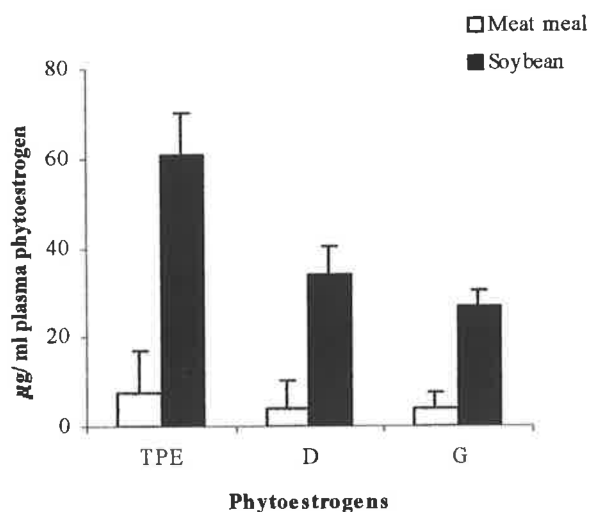


Figure 7.1: Plasma concentration of phytoestrogens in 22 week old Leghorn pullets maintained on either meat meal or soybean diets. Total phytoestrogen, (TPE); daidzein, (D); genistein, (G), mg/day; Columns with different colours (for soybean and meat meal) within the plasma phytoestrogens concentration are differ significantly for TPE (P<0.001), D (P<0.005) and G (P<0.001).

7.3.4. Nutrient retention

The effects of meat meal, soybean meal or synthetic estrogen treatment (17β-estradiol) on nutrient retention are shown in Table 7.5. Dry matter (DM) and other nutrient retention including Ca, Mg and Na (P<0.005), CP and K (P<0.001) increased significantly with meat meal compared with soybean meal, with the exception of P. Treatment with synthetic estrogen appeared to cause a slight fall in most nutrients retention with exception in DM and Na, although the differences were not significant. Finally no significant changes were observed between interaction of diets (meat meal and soybean) and estrogen levels (0 and 50 µg) on DM and all nutrients retention.

Table 7.5: The retention of various nutrients by 22 week old Leghorn pullets fed different diets or treated with different levels of estrogen

	Diets		μg E_2/ kg body weight/day		
	Meat meal	Soybean	0	50	SEM
A. Nutrients retention (%)					
Dry matter (DM)	74.00^{a}	58.80^{b}	65.90	66.80	2.94
Crude protein (CP)	31.60^{a}	9.40^{b}	22.10	18.90	3.05
Calcium (Ca)	28.60^{a}	12.90^{b}	21.60	20.00	3.05
Phosphorous (P)	16.1	11.10	13.70	13.50	2.64
Magnesium (Mg)	31.80^{a}	15.70^{b}	26.40	21.00	3.03
Sodium[2] (Na)	69.80^{a}	53.40^{b}	61.20	62.10	3.25
Potassium (K)	33.10^{a}	15.40^{b}	25.80	22.70	2.99

B. Source of variation	DM	CP	Ca	P	Mg	Na	K
Diets	***	****	***	NS	***	***	****
E_2	NS	NS	NS	NS	NS	NS	NS
Diets x E_2	NS	NS	NS	NS	NS	NS	NS

Mean values on the same row without a common superscript are significantly ***, ($P<0.005$), ****, ($P<0.001$) different; E_2, Estrogen; SEM, Standard error of means;

7.3.5. Egg and egg shell characteristics

Egg and eggshell characteristics are shown in the Table 7.6 (A & B). Egg production (EP), egg mass (EM) and the specific gravity of egg (ESG) were significantly reduced ($P<0.005$; $P<0.001$; $P<0.005$; respectively) by the soybean meal diet in comparison with the meat meal diet. No significant response was observed on egg weight (EW), although there was a slight increase with soybean meal Table 7.6 A. Similarly, estrogen treatment had no effect on egg characteristics.

In terms of eggshell characteristics (Table 7.6 B), no significant differences were observed in the shell weight (SW), shell thickness (ST) and surface area of shell (SFA) with soybean, although all of these parameters, except that of surface area of the shell were slightly reduced by soybean meal in comparison with meat meal. However, the percentage of shell as egg weight (SP), and shell weight per unit area (UW) were decreased significantly ($P<0.05$, $P<0.01$; respectively) by the soybean diet. Most of the shell characteristics except SFA were depressed by estrogen treatment (50 µg/kg body weight/day). There were no significant interactions between diets (meat meal and soybean) and estrogen levels (0 and 50 µg) on the egg and shell characteristics (Table 7.6 C).

Table 7.6: Egg and egg shell characteristics of 18-22 week old Leghorn pullets fed different diets or treated with different levels of estrogen

Egg and egg shell characteristics	Diets		$\mu g\ E_2$ /kg body weight/day		
	Meat meal	Soybean	0	50	SEM
A. Egg characteristics					
Production (EP, egg/hen/house %)	56.8^{a}	34.4^{b}	46.2	45.00	4.98
Weight (EW, g)	43.86	44.76	43.95	44.68	0.99
Mass (E M, g/day)	25.90^{a}	14.30^{b}	19.20	21.10	1.65
Specific gravity (ESG)	1.0781^{a}	1.0744^{b}	1.0767	1.0758	0.001
B. Egg shell characteristics					
Weight (SW, g)	3.70	3.48	3.60	3.58	0.14
Thickness (ST, µm)	303.1	289.6	300.3	292.4	5.65
Surface area (SFA, cm^2)	58.21	59.04	58.30	59.95	1.23
Percentage (SP, %)	8.50^{a}	7.83^{b}	8.24	8.09	0.20**
Weight per unit surface area (UW, mg/ cm^2)	63.65^{a}	59.06^{b}	61.79	60.92	1.62**

C. Sources of variation	Egg characteristics				Egg shell characteristics				
	EP	EW	EM	ESG	SW	ST	SF	SP	UW
Diets	***	NS	****	***	NS	NS	NS	*	**
E_2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Diets x E_2	NS	NS	NS	NS	NS	NS	NS	NS	NS

Means values on the same row without a common superscript are significantly *, (P<0.05), **, (P<0.01), ***, (P<0.005), ****, (P<0.001) different; E_2, Estrogen; SEM, Standard error of means.

7.3.6. Intestinal mucosal morphology

Villus and crypt morphology for different intestinal regions are presented in the Table 7.7. Most villus measurements decreased with the soybean meal diet compared with meat meal diet. Jejunal and ileum crypt depth were significantly reduced ($P<0.001$ and $P<0.005$, respectively) by soybean meal. Also duodenum and ileum height declined significantly ($P<0.05$), with this diet. Ileum surface area showed the same trend in regard to soybean meal.

Although jejunal crypt depth was reduced significant ($P<0.05$) by 50 μg of estrogen compared with the 0 level, it appeared that a small increase in duodenal surface area and villus height are observed in the 50 μg E_2 group. The interaction between meat meal diet and high levels of estrogen (50 μg) was significantly greater in ileum crypt depth ($P<0.005$) and ileum surface area ($P<0.05$) compared with the other interaction groups..

7.3.7. Intestinal enzyme activity

Enzyme activity is illustrated in Figure 7. 2 (A-D). Enzyme activity was examined in the jejunal homogenate tissue in the small intestine. Alkaline phosphatase ($P<0.001$), Ca ($P<0.01$), Mg ($P<0.005$) and Ca/Mg ATPase ($P<0.001$) were reduced significantly by soybean meal compared with meat meal. No changes was identified in Na/K ATPase activity with either soybean and meat meal (Fig. 7.2 A and B)

No differences were obtained with the 0 and 50 μg of estrogen on alkaline phosphatase and most ATPase activity with the exception of Ca ATPase which

significantly increased ($P<0.05$) with estrogen level (50 μg) (Fig. 7.2 C and D). The interaction between diets (meat meal and soybean) and estrogen levels (0 and 50 μg) on enzyme activity were not significant.

Table 7.7: Intestinal mucosal morphometry in 22 week old Leghorn pullets fed different diets or treated with different levels of estrogen

	Diets		µg E_2 /kg body weight/day		
E2/µg weight/day	Meat meal	Soybean	0	50	SEM
A. Crypt dept (CR, µm)					
Duodenum (D)	261.70	267.70	264.20	265.20	9.89
Jejunum (J)	239.70[a]	207.00[b]	234.10[a]	212.50[b]	5.93
Ileum (L)	199.60[a]	173.70[b]	194.10	179.30	5.70
B. Villus height (VH, µm)					
Duodenum	2318.00[a]	2007.00[b]	2159.00	2166.00	98.00
Jejunum	1758.00	1600.00	1681.00	1677.00	60.20
Ileum	1297.00[a]	1145.00[b]	1230.00	1212.00	44.90
C. Villus surface area (VSF, m^{m2})					
Duodenum	0.500	0.449	0.439	0.511	0.039
Jejunum	0.335	0.301	0.318	0.318	0.015
Ileum	0.227[a]	0.198[b]	0.217	0.207	0.009

D. Source of variation	DCR	JCR	LCR	DVH	JVH	LVH	DVSF	JVSF	LVSF
Diets	NS	****	***	*	NS	*	NS	NS	*
E2	NS	**	NS	NS	NS	NS	NS	NS	NS
Diets x E_2	NS	NS	***	NS	NS	NS	NS	NS	*

Mean Values on the same row without a common superscript are significantly *, ($P<0.05$), **, ($P<0.01$), **, ($P<0.005$), ****, ($P<0.001$) different ; E2, estrogen; SEM, standard error of means.

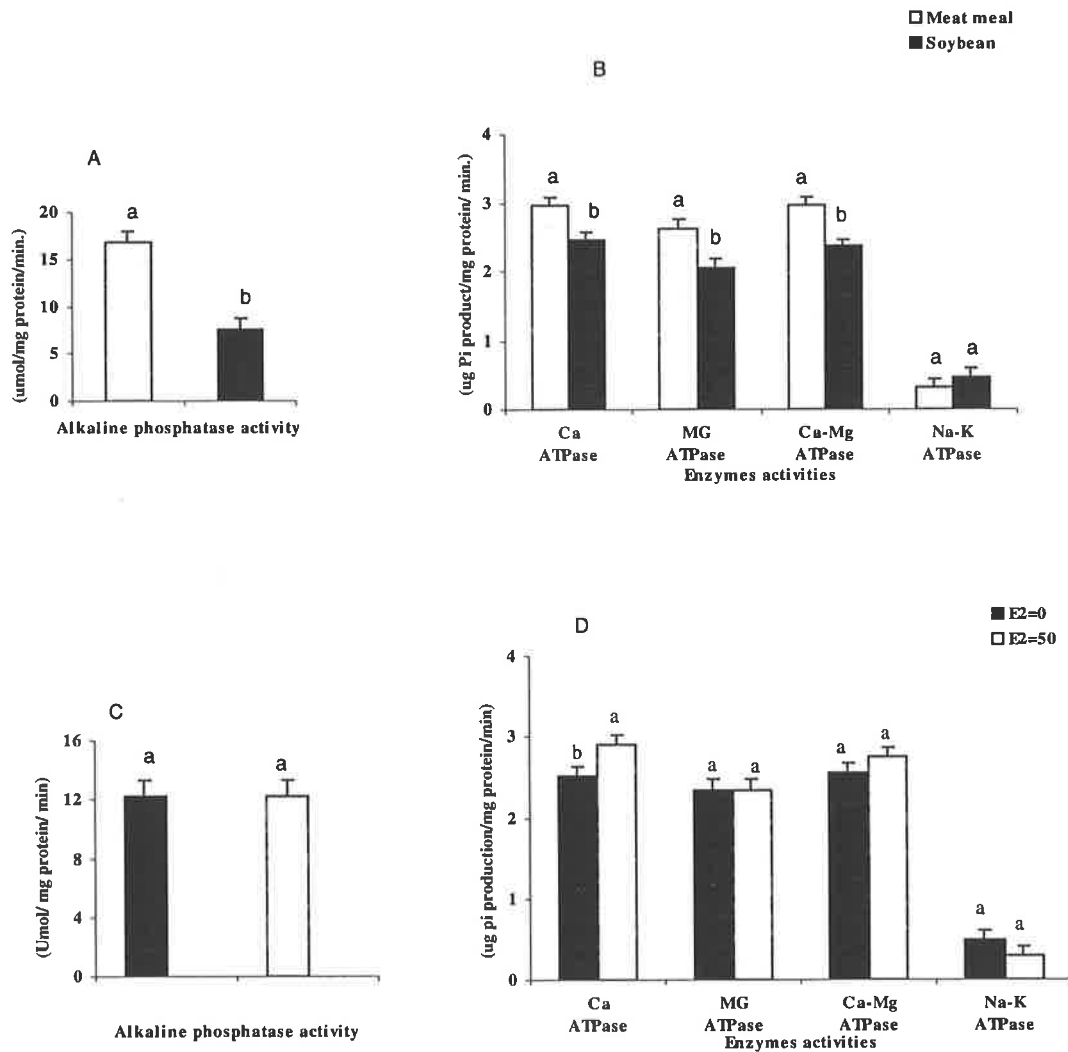
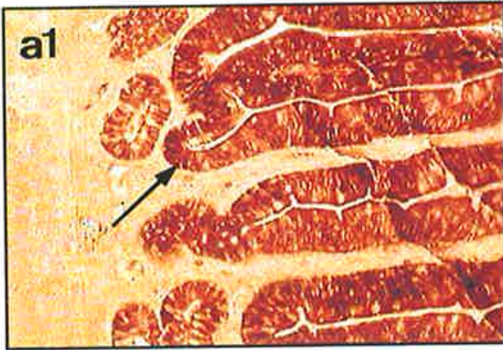
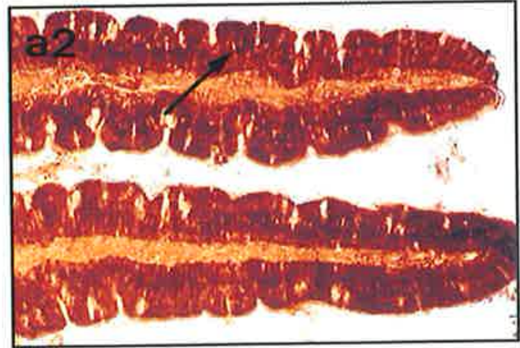
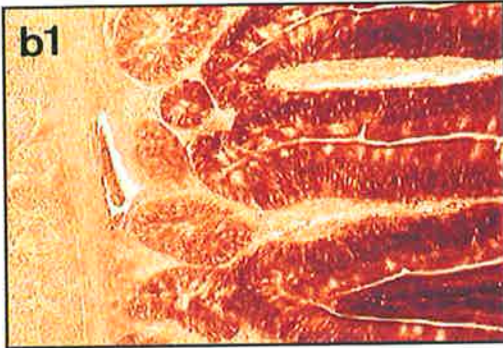
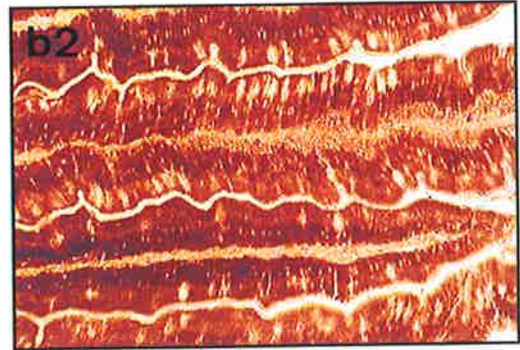
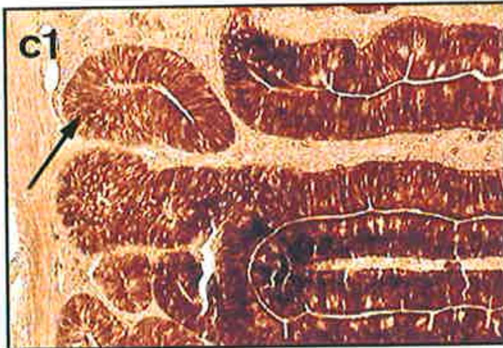
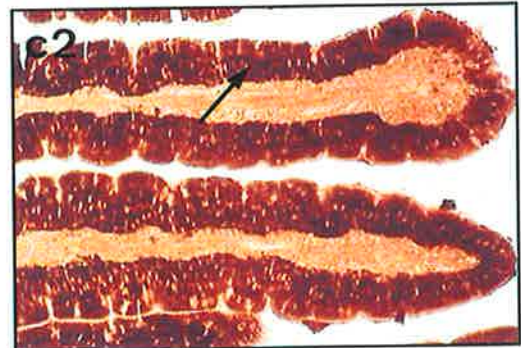
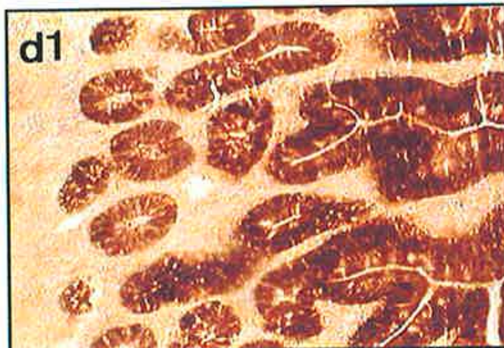
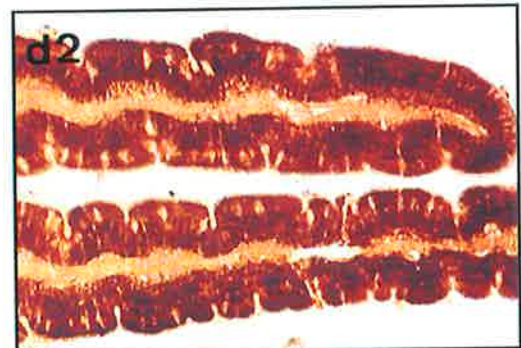


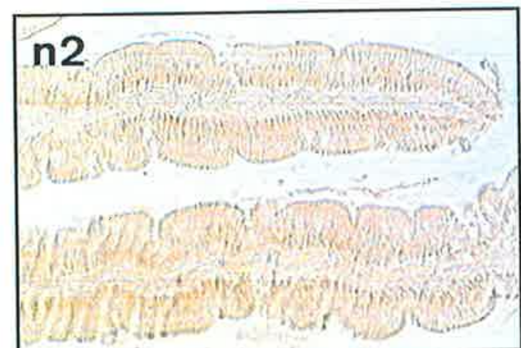
Figure 7.2: Alkaline phosphatase (A) and ATPase enzyme (B) activities in response to meat meal and soybean meal diets; Alkaline phosphatase (C) and ATPase (D), enzyme activities in response to different levels of estrogen (E_2, µg/ kg body weight/day) respectively. For diet effects columns with different letters within the enzyme activity differ significantly for AP ($P<0.001$), Ca ($P<0.01$), Mg ($P<0.005$) and Ca/Mg ($P<0.001$); and for estrogen levels (0 & 50 µg E_2), differ significantly in Ca ($P<0.05$).

7.3.8. Expression of calcium binding protein

The expression of calbindin D_{28k} in the jejunal tissue from the hens maintained on meat meal and soybean diets or dosed with estrogen (0 and 50 μg) are shown in Plate 7.1. No treatment effects were detected in the localisation of calbindin D_{28k} in jejunal tissue. As can be seen from the plate 7.1 the expression of calbindin D_{28k} was localised in the absorptive cell of enterocytes along the length of villus. Overall similar reactions were found to phytoestrogen and synthetic estrogen on calbindin D_{28k} expression in jejunal tissue.

Plate7.1: Immunohistochemical localisation of calbindin D_{28K} in crypt (1) and villus (2) regions of typical jejunal tissue taken from 22 week old Leghorn pullets in response to cereal and soybean meal diets and different levels of exogenous estrogen. Panels a and b represent tissue taken from hens maintained on cereal diet and treated with 0 or 50 µg estrogen/kg body weight/day, respectively. The effect of a soybean meal diet for the same estrogen treatments are represented in panels c and d. Typical negative controls are represented in panels labelled with n. Arrows indicate typical positive stain in epithelial cells.


a1

a2

b1

b2

c1

c2

d1

d2

n1

n2

7.4. Discussion

7.4.1. Growth and feed efficiency

Initial body weight for all treatments, either diet or estrogen level groups, were similar in this experiment. Although meat meal and 50 μg of estrogen decreased weight gain in comparison with other treatments (soybean or nil estrogen), these differences were not significant and similar to the results obtained by Qin *et al.* (1993) and Qin and Klandrof (1993)

There were no significant changes in DFI (g), FI (g/100g body weight) and FCR. Although all of these parameters were depressed to a degree by a high level of synthetic estrogen (50 μg). These data are similar to the results of Robey *et al.* (1988). Overall growth and feed efficiency were similar with respect to diet and level of estrogen.

7.4.2. Growth of the gastrointestinal tract and oviduct

The growth of proventriculus and gizzard (PRG) increased with soybean meal. This increase was reflected in a significantly greater ($P<0.05$) fill capacity with soybean meal than meat meal. No significant effects were observed with soybean meal compared with meat meal in all parameters with the exception of PRG capacity. Intestinal capacity and shell glands weight were slightly increased by soybean meal. In contrast, some reduction appeared in oviduct and liver weight with the soybean diet. As described in the previous chapter, increase in growth, capacity of PRG and also capacity of the small intestine with soybean may be related to the nature of

soybean diet and structure of plant cell wall which could effect the cell proliferation and consequently change the growth and efficiency of these organs (Yu and Chiou, 1996)

Synthetic estrogen, 17β-estradiol, decreased slightly the growth and efficiency of GIT with the exception of liver weight, although these differences were not significant. Increase in liver weight could be due to the effect of synthetic estrogen increasing the liver fat synthesis as elucidated by Qin and Klandrof (1995).

7.4.3. Phytoestrogen intake and plasma phytoestrogen levels

The findings in this area indicated that although no differences appeared in the TPE, D and G intake (mg/g soybean meal/day), TPE, G ($P<0.001$) and D ($P<0.005$) concentration in plasma dramatically increased in those hens fed a soybean meal. As discussed in the chapter 6, despite the absence of phytoestrogens in meat meal or the cereal diet, some of phytoestrogens were found in plasma of hens which were fed these diets. Lien *et al.* (1987) reported that consumption of feed containing more than 1 mg/day phytoestrogen may impair the reproduction of 40-week old hens. This point was demonstrated positively in the current study, because the intake of phytoestrogen in this experiment was much higher than 1 mg/day. Feed intake per unit body weight in 22-week-old hens was greater than 14-week-old pullets, consequently phytoestrogen intake has been increased. Adverse effects may have arisen as a results of this high level of phytoestrogen affecting the estrogen receptor. Calcium deposited in the shell may have been reduced and the reproduction system impaired. The first egg was appeared at third weeks; therefore delay of egg production was 14 days compared with meat meal group. This reduced egg production.

7.4.4. Nutrient retention

The results of this experiment suggested that soybean meal (containing phytoestrogens) and synthetic estrogen (17β-estradiol) reduced nutrient retention with the exception of dry matter which increased slightly with 50 μg of synthetic estrogen. Most nutrients were retained less (less than 50 % in calcium and protein) with soybean meal compared with meat meal. This was significant for DM, CP, Ca, Mg and Na retention. This reduction of nutrient retention particularly calcium may correspond to the declined in egg production and low shell quality which occurred in this experiment. The reason for this effect could be due to the high phytoestrogen in the diet. Large proportion of phytic acid in soybean chelated with calcium and could reduce the availability of calcium (Sebastian *et al.*, 1998). In addition, reduced bioavailability of protein in the soybean by large amount of lectins components that block protein breakdown by proteolytic enzyme in the small intestine could not be ignored (Fuller, 1991), because calcium retention and also absorption is associated by source of protein (Clarence *et al.*, 1995). The adverse effect of synthetic estrogen on nutrient retention may be related to the effect of the chemical on intestinal mucosal, the cause of this aspect still remain unclear.

7.4.5. Egg and egg shell characteristics

The results of this experiment demonstrated that egg characteristics (EP, EM and ESG) but not EW, declined significantly with soybean meal. Delay in egg production (14 days) was also observed in the Leghorn pullets fed soybean meal

compared with meat meal. Egg production also decreased with this diet. Although no significant change occurred with 50 μg of synthetic estrogen on egg characteristics, there were some increases in EW, EM. The reasons for this may related to the high level of phytoestrogen having an adverse effect on the activity and efficiency of the reproduction system and therefore egg production (Lien and Cain 1985). In contrast the results of this experiment with findings of the last experiment may be due to the greater feed intake in the current experiment, consequently increased soybean intake which substantially caused a high level of nutrients inhibitors such as lectins, phytic acid and phytoestrogen as compared with last experiment (using 14 weeks Leghorn pullets).

Significant reduction in SP and UW and non-significant reduction in SW, ST, SFA by soybean meal compared with meat meal resulted in this experiment. There was a similar trend with synthetic estrogen on most shell characteristics. Therefore, the delay in the onset of egg production and also deleterious effects on shell quality by phytoestrogens which have been noted in wild birds and Bobwhite quail (Lien *et al.*, 1985; Lien and Cain 1987) may relate to the possible adverse effects of high phytoestrogen diets.

7.4.6. Intestinal morphology

The results of morphometry measurements of different parameters of villus and at different regions of the intestine were noted. Crypt depth of jejunum and ileum, villus height of duodenum and villus surface area of ileum were reduced significantly by soybean compared with meat meal diet. Although the structure of the mucosal in the small intestine of growing chicken (before and after hatching) due to diet has

been tested Uni *et al.* (1995). Yu and Chiou (1996) reported that high dietary fibre could cause larger intestinal villi in the chicken, but the relationship between mucosal structure of the small intestine and diet particularly soybean, in laying hens has not been noted by others. Therefore the reduction in most parameters of villus and intestinal regions with soybean may be due to damage caused by a villus parameters by slightly higher fibre component in soybean diets as described for rabbits by Yu and Chiou, (1996).

As can be seen from the Table 7.7 no significant differences were observed in villus crypt morphology in response to a high level of estrogen (50 μg), except in jejunum crypt depth. There was a greater significant interaction between meat meal diets and high level of estrogen on crypt depth ($P<0.005$) and surface area ($P<0.05$) of the ileum compared with the other treatments. This characteristic could be dependent on some factors such as phytic acid (Sebstian *et al.*, 1998) lectins (Fuller, 1991) and also phytoestrogens (Lien *et al.*, 1985) or other unknown agents that inhibit availability of nutrients, which are absence in the meat meal. No report has been noted in this aspect.

7.4.7. Intestinal enzyme activity

The results of measurement of enzyme activity in this study demonstrated that the activity of alkaline phosphatase and ATPase (Ca, Mg and Ca/Mg ATPase) in jejunal homogenate were reduced significantly by soybean meal compared with meat meal with no change in Na/K ATPase. Although there have been no reports concerning soybean on the activity of these enzymes, it seems these adverse effects were related to the high level of phytoestrogens in soybean meal compared with meat meal diets.

Particularly the result from alkaline phosphatase activity regarding soybean effect is different with the last experiments (14-week-old leghorn pullets). The daily feed intake in this experiment (18-22 week Leghorn pullets) is higher than the last experiment (10-14 week old Leghorn pullets) Table 6.2 and 7.2. Therefore this leads a to increase soybean intake in this experiment table 7.4. Consequently there is a greater daily intake of phytoestrogen resulting in a higher plasma phytoestrogen concentration in 22 weeks compared with 14 weeks old Leghorn pullets. This could be the main reason for lower enzyme activity in the current experiments. In addition high intake of soybean which is rich in lectins components may inhibit protein breakdown by intestinal proteolytic enzyme and leading to unavailability of sufficient protein for enzyme activities (Fuller, 1991; Clareance *et al.,* 1995).

A similar activity was found in alkaline phosphatase at low (0) and high level (50) of synthetic estrogen, while increasing effects were observed in ATPase (Ca, and Ca/Mg ATPase) activity with synthetic estrogen (50 μg). In addition there was significantly greater effect ($P<0.05$) in Ca ATPase activity at high level of synthetic estrogen compared with low levels, as a demonstrated by Qin and Klandorf (1993) and Picotto *et al.* (1996).

The interaction of a high level of estrogen and meat meal were significantly higher in Ca/Mg ($P<0.01$) and Na/K ATPase ($P<0.05$) activity compared with interaction between of soybean meal and estrogen level. As mentioned above, these particular enzyme activities in jejunal tissues may be associated with availability of some nutrient such as protein. In contrast the interaction between 0 μg of estrogen and meat meal on Mg ATPase was significantly higher ($P<0.005$) than in other treatments groups. This reflects one of the reasons for high retention of calcium with meat

meal, as Roberts and Brackpool (1994) have shown, Mg can effect calcium transport during the shell formation. They have proposed that the magnesium can act as a co-factor role for calcium transport in shell gland during the shell formation. It has been found that the majority of ATPase-dependent calcium transport in the shell gland can only occur with presence of Mg. Therefore the high retention of Mg in this case may increase Mg ATPase and consequently Ca absorption.

7.4.8. Expression of Calcium binding protein (calbindin D_{28k})

Based on the results of this experiment, there were no dramatic differences in the localisation of calbindin D_{28k} in the jejunum tissue in regard to exogenous estrogen, phytoestrogens diet or their interactions. The expression of calbindin D_{28k} is localised in the epithelial cells the along the length of the villus as described by Jande *et al.* (1981) and Bar *et al.* (1990). Others have, not considered the expression of calbindin D_{28k} in jejunal tissue of the small intestine in the current experiment in particular, for phytoestrogens.

7.4.9. Conclusion

Similar results were obtained in WG (g), DFI (g), FI (g/100g body weight) and FCR related to the effect of diets (meat meal and soybean meal) and estrogen level (0 and 50 μg). No significant differences in initial body weight could lead to these results. Although the capacity of PRG was significantly higher with soybean meal, no significant differences were identified in growth of PRG, OW, SGW, LW as well as intestinal capacity (SIC). The reason for increased capacity of PGR may relate to the nature of the soybean diet.

No differences were recognized in TPE, D and G intake, mg/day, in regards to different levels of estrogen (0 and 50 μg). In contrast there was a high concentration of phytoestrogens (TPE, D and G) in plasma of those groups of pullets fed soybean compared with those hens fed meat meal. No phytoestrogen was detected in the meat meal diet.

Significant effects were achieved in the reduction of the retention of most nutrients with soybean meal compared with meat meal. This may have been due to the availability of nutrients in meat meal and also possible presence of some stimulatory effectors in the meat meal or adverse effectors in soybean.

In terms of egg and eggshell characteristics, EP, EM, SEG, SP and UW were significantly reduced by soybean meal, although no significant changes were noted related to synthetic estrogen on egg and shell characteristics. The main reason for the reduction of egg production (EP) and shell quality can be related to the inhibitory effects of phytoestrogens on the reproduction system as emphasised by Lien and Cain (1987), since diets have been balanced in terms of energy, protein, amino acids, minerals and sufficient vitamins. However the adverse effects of the other inhibitors in the feed, particular soybean, can not be ignored in this particular case. Although the adverse effect of phytoestrogens on reproduction system in 40 week old hens have been emphasised by others (Liren and Cain 1985) the current study has identified the different phytoestrogens (D and G) associated with this action on Leghorn pullets.

Reduction of the ATPase enzyme activity, in particular Ca ATPase activity, with soybean meal containing phytoestrogens contributed to the reduction in egg production and eggshell quality. The decrease of crypt depth and villus height in the small intestine by soybean meal may be the major reason for the adverse effects of soybean in reducing nutrient retention in particular Ca absorption.

A delay in the onset of egg production and the predominantly negative effect on EP and eggshell quality was observed when pullets were fed soybean meal in this experiment. It is clear, based of these findings that the poultry industry may have to determine the permissible intake of soybean meal in commercial laying hen diets.

CHAPTER EIGHT

GENERAL DISCUSSION

General discussion

Egg as a source of energy and minerals can be a very important source of nutrients in human diets. Despite the high level of cholesterol in egg yolk which has caused egg consumption to decline particularly in the western society, eggs still play a vital role in providing many nutrients as a food or in processed foods. Eggs are used in many processed products, e.g. various fruit juices, particularly apple juice, is common in New York (the name chosen for this product is Tren a combination between tree and hen. It has been suggested that Tren should replace present breakfast meals, Sim and Nakai (1994). In addition, eggs and the potential of growth and development of the egg embryo is a major reason for growth of the poultry industry. Therefore the survival of this industry is dependent on egg production and egg quality. For these reasons eggs need to be protected from physical and pathological agents between production and consumption by a well-formed eggshell. The major function of the shell is to maintain the quality of the egg. Poor shell quality can lead to losses of approximately US $ 500 million in the annual global egg production (Etches, 1996). Shell quality can be maintained by minerals, particular by calcium carbonate, since this compound constitutes up to 90% of the shell composition. The supply of calcium carbonate is positively dependent on feed calcium, although calcium requirement is a function of calcium retention, egg production, shell quality, age and hormonal status.

It is well established that the calcium requirement of laying hens increases dramatically during the sexual maturity and at onset of lay (Wu *et al*, 1994). The requirement of hens for calcium is the highest of all domestic animals (Austic and Nesheim, 1990). In view of this fact, laying hens require two grams of calcium to

form the shell of each 60g egg. This high demand for calcium in laying hens is affected by vitamin D_3 ($1,25(OH)_2 D_3$) during the process of calcium transport in the laying cycle (Wu *et al.,* 1994). Calcium transport in the small intestine is regulated by the hormonal status, during the sexual maturity. It has been suggested that the production of vitamin D_3 ($1,25(OH)_2D_3$), a regulator of calcium metabolism in the kidney by 1-hydroxylase enzyme, is affected by estrogen (Hurwitz, 1989; Wu *et al.,* 1994).

The direct effect of estrogen on calcium transport in laying hens, particularly in pre-laying hens, remains unclear. The question investigated in this study, was whether there is a possibility that the direct effect of estrogen modulates the proteins associated with calcium transport (the calcium binding protein, calbindin D_{28k}, ATPase and alkaline phosphatase) in the intestinal tissue during the sexual maturation. Feed efficiency, growth, egg and egg shell characteristics, enzyme activity, intestinal mucosa structure and expression of calbindin D_{28k} in relation to the effect of synthetic estrogen (17-β estradiol) and diets with high levels of plant estrogenic components (phytoestrogens) were also tested in this study. Since, soybean (a major component of poultry diets) contains phytoestrogens, pre-laying hens were fed diets containing soybean meal.

The first study was designed to determine the temporal and spatial expression of estrogen receptors (ER) and calcium binding protein (calbindin D_{28k}) in jejunal tissue of Isa-brown hens (15 and 26 weeks old). The calbindin D_{28k} expressed in the jejunal cytoplasm have been described by Jande *et al.* (1981), Bar *et al.* (1990) and Wu *et al.* (1994). The results in the current study demonstrated that the localisation of the estrogen receptor (ER) preceded calbindin D_{28k} expression. The expression of ER

regardless of the age of hens was detected in the cytoplasm of epithelial cells in the villus tip enterocytes of jejunal tissue (the first time in hens) although this has been recorded in the human intestine (Arjmandi *et al.*, 1993). The findings demonstrated the possible existence of estrogen receptors in the intestine, particularly the jejunum which is a large and sensitive site of small intestine for calcium absorption (Hurwitz, 1989) and showed the potential of estrogen to regulate this intestinal tissue. Indeed Wu *et al.* (1994) have shown that most calcium transport occurred in the tip of the villus. Therefore the increase in estrogen level during the sexual maturation may induce calbindin D_{28k} expression or may has the non-genomic effect on ATP cycle (Picotto *et al.*, 1996) and also increase the absorptive capacity for calcium. It is believed that vitamin D receptors are located in the both crypt and villus enteroctyes of pre-laying hens (Wu *et al.*, 1994). Also it has been noted that the expression of calbindin D_{28k} observed in the cytoplasm and nuclei of absorptive cell in the enterocytes of the chicken intestine Lawson (1978). The interaction of vitamin D receptors and estrogen receptors which have been detected in the current study, may induce the synthesis of calbindin D_{28k} for regulation of calcium transport in the pre-laying. (Krust *et al.,* 1986; Wu *et al.*, 1993 and 1994).

In addition to ER and calbindin D_{28k} expression, the activity of alkaline phosphatase significantly increased at 26-week old hens compared with 15-week old pullets (non-egg production). This agreed with the results obtained by Nys and Laage (1984). The significant increase in villus surface area in the duodenum and also villus height could be associated with the enhancement in calcium absorption in this region. The question arises however, whether there is an effect of synthetic estrogen on calcium absorption during the sexual maturity in a common Australian strain of hens (Tegel).

The second study was carried out to compare the effect of synthetic and endogenous estrogen on calcium absorption and consequently on egg and eggshell characteristics. The results obtained in this experiment (with 18-22 week old Tegel pullets) showed that there was no response to different levels of estrogen (0, 10 and 100 μg/kg body weight /day) on feed intake, weight gain and FCR. Such findings have been observed by others (Adams *et al.*, 1950; Qin and Klandorf, 1993).

The retention of calcium and the activity of alkaline phosphatase and ATPase enzymes, except that of Na/K ATPase, were reduced by 10 μg/kg body weight/day of E_2 Also, no differences were observed in morphometry of different regions of the small intestine and calbindin D_{28k} expression between treatment groups. In contrast an increase of 10% in the shell thickness and 6.5% in shell weight per unit area by this level of E_2 was observed. These may be due to changes in both the architecture of the ultrastructure of shell and an increase in calcium absorption. An increase in the retention of protein could be associated with this increase in calcium absorption (Civitelli *et al.*, 1992). There is still a doubt that the particular reaction recorded in this case may be specific to the strain used (Tegel). This question led to conducting another experiment to investigate the effect of strain (genetic potential) and level of calcium and estrogen on calcium retention and consequently on egg and egg shell characteristics.

The aim of a third study was to compare 18-22 weeks old Tegel with Leghorn pullets. In terms of growth and feed efficiency, increasing the dose of estrogen reduced body weight, as described by Sommerville *et al.* (1989). No response was found with respect to high and low levels of calcium in growth and feed efficiency.

However it appeared that a significant increase occurred in daily feed intake and a reduction in FCR in Tegel pullets compared with Leghorn pullets. These reactions could be related to the higher initial body weight of Tegels and also the greater genetic potential of Leghorn to increased feed conversion.

The retention of calcium was significantly higher at a low level of dietary calcium in comparison with a high level; similar results were obtained by Gilbert (1983) and Hurwitz (1989). Tegel pullets showed more response in calcium retention compared with Leghorn pullets. This could be related to the higher initial body weight of Tegels and also increased in protein retention which is known to be associated with enhance calcium retention and absorption (Civitelli and Avioli, 1994). In contrast, the growth rate of Leghorn pullets 5.25 g/kg body weight/ day was higher than Tegel (4 g).

The result of this study demonstrated that Leghorn pullets have a greater ability to improve shell quality (shell thickness, shell weight and percentage of shell) than Tegel pullets. In contrast, high calcium retention, better egg characteristics and more enzyme activity were observed by Tegel pullets. These findings suggest there is an interaction between calcium and other physiological response, since reduction in calcium retention, Ca ATPase activity and subsequent plasma calcium may lead to increase the activity of PTH (Elaroussi *et al.*, 1994). This could increase plasma calcium and regulate calcium metabolism. Calcium metabolism is a complex relationship between kidney, shell gland, bone, small intestine and blood stream during the shell formation. This relationship may be stronger with the high rate of growth in Leghorn than Tegel pullets. The genetic potential of Leghorn pullets and the contribution of some intracellular organelles in calcium transport should not be

ignored in this relationship.

Although the interaction between 50 µg of E_2 and a high level of calcium with Leghorn pullets was not significantly greater than in the others treatments, large differences were identified in the shell thickness 411.2 µm and shell weight 5.51 g which were greater 23 µm and 0.5 g respectively compared with the other treatments groups. These finding demonstrated that Leghorns with a high potential in egg shell quality and more response to estrogen level were the most useful strain for the next experiment to determine the effect of plant estrogen components (phytoestrogen of soybean) on laying hens.

The fourth study was aimed at determining the effect of plant estrogenic compounds (phytoestrogens) on calcium retention before the onset of lay (10-14 weeks) in Leghorn pullets. Further was aimed at testing the switching on of calbindin D_{28k} expression with phytoestrogen and synthetic estrogen (50 µg) which showed a greater response to shell quality in Leghorns than Tegels in the previous study.

The results demonstrated that soybean (as a phytoestrogen source) and meat meal on 50 µg of estrogen had similar effects on feed intake. Weight gain and feed conversion were significantly reduced by soybean compared with meat meal. This could be related to the high level of phytoestrogens in soybean (daidzien and genistein) (Lien and Cain, 1987) and also to the cell wall content of soybean, since it is known that a considerable proportion of some constituents in legumes are not digested and absorbed (Fuller, 1991; Sebsastian *et al.*, 1998). Calcium in particular is bounded to phytic acid and this reduces its ability to digest and absorbed in poultry

(Sebsastian *et al.*, 1998). Significant differences were found in plasma concentration of the phytoestrogens of those hens fed soybean meal compared with meat meal, chiefly because no phytoestrogens were identified in meat meal or the cereals based diet. The existence of phytoestrogen in plasma of those hens fed meat meal was probably related to the presence of phytoestrogen precursors in diets which are not detectable until metabolised by the hens (personal communication, Dalais, 1998)

Nutrients retention, particularly calcium retention, increased dramatically with the soybean meal diet and a high level of E_2 (50 μg). In contrast, ATPase enzyme activity, mucosa structure of small intestine were reduced by soybean meal. More expression of calbindin D_{28k} was observed by this diet in comparison with meat meal. The increase in protein retention, alkaline phosphatase activity and a physiological requirement for storage calcium in 10-14 weeks old of Leghorn pullets were the main reasons for high calcium retention in this case (Gibert, 1983; Van Os, 1987; Hurwitz, 1989; Civitelli and Avioli, 1994). The results of this experiment demonstrated that pre-laying Leghorn pullets at 10-14 weeks of age attempt to alter the calcium pool for their maintenance and to store calcium bone for egg and egg shell production for the next stage of growth (the onset of lay). The result of this experiment suggests that the principals involve in relation to the effect of phytoestrogen on calcium retention during the egg production and shell formation are still unclear and need to be clarified. Therefore a further study was designed to address in these issues.

In the fifth study 18-22 weeks old Leghorn pullets fed soybean and meat meal diets and the administration of estrogen (0 and 50 μg) were tested to determine the beneficial or adverse effect of phytoestrogen on egg production and shell quality.

The results of this study indicated that there were no responses in weight gain, feed intake and FCR regarding either diet (soybean and meat meal) or synthetic estrogen. Such effects was also noted by Robey *et al.* (1988) and Qin and Klandrof (1993). No differences were found in growth of the proventriculus, oviduct weight, shell gland weight, liver weight as well as small intestine capacity with respect to treatments groups, but increases in the proventriculus and gizzard capacity may be related to the nature of soybean.

High levels of total phytoestrogen, daidzein and genistein concentrations in plasma of those hens fed soybean compared with meat meal, chiefly because no phytoestrogen was detected in meat meal.

Reduction in nutrient retention, particularly calcium, during consumption of soybean meal compared with meat meal, may be due to the strong chelating of calcium with phytic acid. In addition, calcium forms a wide variety of insoluble products with some legumes such as soybean and becomes unavailable (Sebastain *et al.*, 1998). This reduction also corresponds to the decline in alkaline phosphatase and ATPase activity which parallels the reduction of intestinal mucosa structure. No changes in expression of calbindin D_{28k} in jejunal tissue occurred with respect to soybean treatment. These were reflected in the adverse effects observed in egg production and eggshell characteristics.

In terms of egg production and shell quality, egg mass, specific gravity, shell percentage of egg and weight per unit area of shell were significantly reduced by soybean meal compared with meat meal. No significant differences in these

measurements occurred by treatment with synthetic estrogen. A delay in egg production also occurred by the phytoestrogen. This is in agreement with the results of Lien *et al.* (1985) and Lien and Cain (1987). The permissible amount of phytoestrogen in the soybean meal as one of the base ingredients in commercial laying hens diet needs to be clarified.

In conclusion, the temporal and spatial expression of the estrogen receptor in the jejunal tissue at 15 and 26 weeks old hens demonstrated that this expression precedes calbindin D_{28k} synthesis. In view of this fact, estrogen may have the potential to modulate calbindin D_{28k} and consequently increase calcium absorption during the egg production and shell formation. Non-genomic effect of estrogen receptors on Ca ATPase also should be noted in this respect. This hypothesis led, to the examination of the effect of synthetic estrogen on calcium retention and shell quality in Tegel pullets. Improvement in shell thickness and shell weight per unit area were found to occur as a results of this treatment. Considerable effort has been made in understanding the effect of different strains of hens on calcium retention and eggshell quality. This question lead to compare Tegel with Leghorn pullets. The experimental results showed that Leghorn pullets at the onset of lay responded to high dietary calcium (4%) and 50 μg estrogen. These findings resulted in investigating the effects of plant estrogens in soybean on calcium retention and eggshell quality in Leghorn pullets. In 14-week old Leghorn pullets (before onset of lay) high calcium retention was observed with respect to high protein retention and more alkaline phosphatase enzyme activity. In contrast, during egg production and shell formation in 22 weeks old Leghorn pullets, low calcium retention, delay of egg production and poor shell quality were noted in hens maintained on a soybean diet. In addition to the other causes in regard to these points, a high intake of soybean (and

consequently a greater phytoestrogen intake) in 18-22 week old Leghorn pullets (compared with 10-14 week old) caused a high plasma concentration of phytoestrogen (daidzein and genistein). This could impair the reproduction in hens as emphasised by Lien and Cain (1987).

The poultry industry needs to be warned of the effect of high levels of phytoestrogens in soybean. More attention need to be taken of this potentially serious problem, as these compounds may cause a reduction in egg production with a decline in reproduction capacity, a delay of egg production and poor egg shell quality. This reaction may occur since phytoestrogens act as inhibitors to the synthesis of particular protein associated with calcium transport. It is urgent that the poultry industry determines the permissible amount of phytoestrogens as they can act as anti-nutritive factors and lead to reduced performance inlaying hens and consequently huge economical losses. There is need for further investigations into the action of other functions impacting on the effects of phytoestrogens on the performance of laying hens.

REFERENCES

Adams, J. L., McGbbon, W. H. and Casida, L. E. (1950). The effect of orally administered synthetic estrogen on single comb white Leghorn pullets. *Poultry Science*. **29**, 666-671.

Akerfeldt, K. S., Coyne, A N., Wilk, R. R., Thulin, E. and Linse, S. (1996). Ca^{2+}-binding stoichiometry of calbindin D_{28k} as assessed by spectroscopic analyses of synthetic peptide fragments. *Biochemistry*. **35**, 3662-3669.

Alberts, B., Bray, D., Lemis, J., Raff, M., Roberts, K. and Watson, J. D. (1994). The cell-division cycle. In: Anonymous *Molecular biology of the cell.* Third ed. pp. 863-910. New York: Garland.

Ambrecht, H .J., Doubek, W. G. and Poter, S. B. (1988). Calcium transport by basolateral membrane vesicles from rat small intestine with age. *Biochemica Biophysica Acta*. **944**, 347-373.

Arjmandi, B. H., Salih, M. A., Herbert, D. C., Sims, S. H. and Kalu, N. (1993). Evidence for estrogen receptor-link calcium transport in the intestine. *Bone and Mineral*. **21**, 63-73.

Austic, R. E. and Nesheim, M. C. (1990). *Poultry production.* p. 325. 13th edn. Phiadelphia: Lea & Febgir.

Baghurst, P. A. (1997). Phytoestrogen and hormone dependent cancer. **21**, p. 98. Brisbane, Queensland, Australia: The Nutrition Society of Australia.

Balnave, D. (1993). Influence of saline drinking water on eggshell quality and formation. *Would's Poultry Science.* **49**, 108-119.

Bancroft, J. D and Cook, H. D. (1984). *Manual of histological techiques.* p.274. London: Churchill Livingstone.

Banerjee, G. C. (1992). *Animal husbandry*, p. 854. seven ed. Oxford and IBH.

Bar, A., Cohen, A., Eisner, U., Risenfeld, G. and Hurwitz, S. (1978). Differential response of calcium transport system in laying hens to exogenous and endogenous changes in vitamin D status. *Journal of Nutrition*. **108**, 1322-1328.

Bar, A. and Hurwitz, S. (1972). Relationship of duodenal calcium-binding protein to calcium absorption in the laying fowl. *Comparative Biochemistry and Physiology*. **41A**, 383-389.

Bar, A. and Hurwitz, S. (1979). The interaction between dietary calcium and gonadal hormones in their effect on plasma calcium, bone, 25-hydroxycholecalciferol-1-hydroxylase, and duodenal calcium binding protein, measured by a radioimmunoassay in chicks. *Endocrinology*. **104**, 1455-1460.

Bar, A. and Hurwitz, S. (1987). Vitamin D metabolism and calbindin (calcium-binding protein) in aged laying hens. *Journal of Nature.* **117**, 1775-1779.

Bar, A., Striem, S., Rosenberg, J and Hurwitz, S.(1988). Egg shell quality and cholecalciferol metabolism in aged laying hens. *Journal of Nutrition.* **118,** 1018-1023.

Bar, A., Shani, M., Fullmer, C. S., Brindak, M.E. and Striem, S. (1990). Modulation of chick intestinal and renal calbindin gene expression by dietary vitamin D_3, 1,25-dihydroxyvitamin D_3, calcium and phosphorus. *Molecular and Cellular Endocrinology.* **72**, 23-31.

Bar, A., Striem, S., Mayel-Afshar, S. and Lawson, D.E.M. (1990). Differential regulation of calbindin-D_{28k} mRNA in the intestine and eggshell gland of laying hen. *Journal of Molecular Endocrinology* **4**, 93-99.

Baski, S. N. and Kenny, A. D. (1981) Vitamin D metabolism in aged Japanese quail: dietary calcium and estrogen effects. *American Journal of Physiology* **241**, E275-E280.

Beato, M. (1989) Gene regulation by steroid hormones. *Cell.* **56**, 335-344.

Bennetts, G., Underwood, E. J. and Shier, F. L. (1946) A specific breeding problem of sheep on subterranean clover pastures in western *Australia. Australian Journal of Agricultural Research.* **22**, 131-138.

Berry, W. D. and Barke, J. (1990) Research note: Induced molt increases eggshell quality and calbindin-D_{28k} content of eggshell gland and duodenum of aging hens. *Poultry Science* **70**, 655-657.

Bickoff, E. M. (1959) Determination of estrogenic activity in fresh and dried forage. *Journal of Animal Science* **18**, 1000-1009.

Bikle, D. D., Munson, S. and Chafouleas, J. (1984) Calmodulin may mediate 1,25-dihydroxyvitamin D-stimulated intestinal calcium transport. *European Biochemical Societies* **174**, 30-33.

Boyle, I. T., Miravet, L., Gray, R. W., Holick, M. F. and Deluca, H. F. (1972) The response of intestinal calcium transport to 25-hydroxy and 1,25-dihydroxyvitamin D in nephrectomized rats. *Endocrinology.* **90**, 605-608.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Brasitus, T. A., Dudeja, P.K., Eby, B. and Lau, K. (1986). Correction by 1,25-dihyroxycholecalciferol of the abnormal fluidity and lipid composition of enterocyte brush border membranes in vitaminD- deprived rats. *Journal of Biological Chemistry* **261**, 16404-16409.

Brody, T. (1994). *Nutritional biochemistry.* p. 658. Berkeley California: Academic press INC.

Broke, J. L., Caride, A., Verma, A. K., Penniston, J. T. and Kumar, R. (1990) Cellular and segmental distribution of Ca^{2+}-pump epitopes in rat intestine. *Pfluger Archives European Journal physiology* **417**, 120-122.

Broke, J. L., Minami, J., Verma, A. K., Penniston, J. T. and Kumar, R. (1987) Monoclonal antibodies to human erythrocyte membrane Ca^{2+} Mg^{2+} adenosine triphosphatase pump recognise an tubule cells. *Journal of Clinical Investigation* **80**, 1225-1231.

Brommage, R., and Deluca, H. F. (1984). A maternal defect is responsible for growth failure in vitamin D-deficient rat pups. *American Journal of physiology.* **246**, E216-E220.

Bronner, F. (1991). calcium transport across epithelia. *International Review of Cytology.* **131**, 168-212.

Bronner, F. (1992). Current concepts of calcium absorption: An overview. *Journal of Nutrition.* **122**: 641-643.

Bronner, F., Pansu, D. and Stein, W.D. (1986). An analysis of intestinal calcium transport across the rat intestine. *American Journal of Physiology.* **250**: G561-G569.

Bruns. M. E., Overpeck, J. G., Smith, G. C., Hirsch, G. N., Mills, S. E., and Bruns, D. E. (1988). Vitamin D-dependent calcium binding protein in the rat uterus; differential effect of estrogen, tamoxifen, progesterone, and pregnancy on accumulation and cellular localisation. *Endocrinology.* **122**, 2371-2378.

Brunsgaard, G. and Eggum, B. O. (1995). Small intestinal tissue structure and proliferation as influenced by adaptation period and indigestible polysaccharides. *Comparative Biochemistry and Physiology.* **112A**, 365-377.

Brunsgaard, G., Eggum, B. O. and Sandstrom, B. (1995). Gastrointestinal growth in rat as influenced by indigestible polysaccharides and adaptation period. *Comparative Biochemistry and Physiology.* **11A**, 369-377.

Carafoli, E. (1991). Calcium pump of the plasma membrane. *Physiology Review.* **70**, 129-153.

Castaldo, J. D. and Maurice, D. V. (1989). Shell gland adenosine triphosphatase in hens producing strong and weak egg shells. *British Poultry Science.* **31**, 225-229.

Castillo, L., Tanaka, Y., Deluca, H. F. and Sunde, M. L. (1976). The stimulation of 25-hyroxyvitamin D_3-1a-hydroxylase by estrogen. *Archives of Biochemistry and Biophysics.* **179**, 211-217.

Castillo, L., Tanaka, Y., Wineland, M. J., Jowsey, J.O. and Deluca, H. F. (1979). Production of 1, 25-hydroxy vitamin D_3 and formation of medullary bone in the egg laying hens. *Endocrinology.* **104**, 1598-1601.

Celio, M. R., Pauls, T. L. and Schwaller, B. (1996). Introduction to EF-hand calcium-binding proteins. In: Celio, M.R. (Ed.). *Guidebook to the calcium-binding proteins.* pp. 1-15. New York: Oxford University Press.

Chen, S. W., Francis, B. M. and Dziuk, P. J. (1993). Effect of concentration of mixed-function oxidase on concentration of estrogen, rate of egg lay, eggshell thickness and plasma calcium in laying hens. *Journal of Animal Science.* **71**, 2700-2707.

Chen, S. W., Dziuk, P. J. and Francis, B. M. (1994). Effect of four environmental toxicants on plasma Ca and estradiol 17β and hepatic P450 in laying hens. *Environmental Toxicology and Chemistry.* **13**, 789-796.

Christie, W. W. and Noble, R. C. (1984). Recent developments in lipid analysis. In: Lawerence, J.F. (Ed.). *Food constituents and food residues.* pp. 1-30. New York: Marcel Dekke.

Civitelli, R. and Avioli, L.V. (1994). Calcium, phosphate and magnesium absorption. In: Johson, L. R. (Ed.). *Physiology of the gastrointestinal tract.* pp. 2173-2181. New York: Raven Press.

Civitelli, R., Villareal, D. T. and Avioli, L. V. (1992). Dietary L-lysine and calcium metabolism in human. *Nutrition* **8**, 400-408.

Clarence, B. A., Baker, D. H and Lewis, A. J. (1995). *Bioavailability of nutrients for animals amino acids, minerals and vitamins.* p 439. New York: Academic Press.

Coward, L., Barnes, N. C., Setchell, D. R. and Barnes, S. (1993). Genistein, Daidzein and their B-glycoside conjugates: Antitumor isoflavones in soybean foods from American and Asian diets. *Journal of Agricultural and food chemistry.* **41**, 1961-1967.

Dalton, C. (1985). *An introduction to practical animal breeding.* pp. 48-60. London: sons & Co.LTd.

Darnell, J., Lodish, H. and Baltimore, D. (1990). Plasma membranes. In: Darnell, J., Lodish, H. and Baltimore, D. (Ed.) *Molecular cell biology.* pp. 491-582. US Scientific American Bool.

Davis, W. L., Jones, R. G. and Hagler, H. K. (1979). Calcium containing lysosomes in the normal chick duodenum: A biochemical and analytical electron microscopi study. *Tissue and Cell* **11**, 127-138.

Delorme, A. C., Danan, J. L., Acker, M. G., Ripoche, M. A. and Mathiru, H. (1983). In rat uterus 17 beta-estradiol stimulate a calcium- binding protein

similar to the duodenal vitamin D-dependent calcium-binding protein. *Endocrinology*. **113**, 1340-1347.

Deluca, H. F. (1979). The vitamin D system in the regulation of calcium and phosphorus metabolism. *Nutrition Review*. **37**, 161-193.

Deluca, H. F. (1980). The control of calcium and phosphorus metabolism by vitamin D endocrine system. In: Levander, O.A. and Chang, L. (Ed.) *Micro nutrient interactions: vitamins, minerals and hazardous elements.* pp. 1-17. New York: Academic of Science.

Dostal, L. A. and Toverud, S. U. (1984). Effect of vitamin D_3 on duodenal calcium absorption in vivo during development. *American Journal of Physiology*. **246**, G528-G534.

Eastin, W. C. and Spaziani, E. (1978). On the mechanism of calcium secretion in the avian shell gland (uterus). *Biological of Reproduction*. **19**, 505-518.

Elaroussi, M. A., Forte, L. R., Eber, S. L. and Biellier, H. V. (1993). Adaptation of kidney during reproduction: role of estrogen in regulation of responsiveness to parathyroid hormone. *Poultry Science*. **72**, 1548-1556.

Elaroussi, M. A., Forte, L. R., Eber, S. L. and Biellier, H.V. (1994). Calcium homeostasis in the laying hen. 1. Age and dietary calcium. *Poultry Science*. **73,** 1581-1589.

Etches, R. J. (1996). *Reproduction in poultry.* p 318, Ontario: CAB International.

Evans, M. (1985). *Nutrient composition of feedstuffs pigs and poultry.* p. 134. Brisbane: department of primary industry.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science*. **240**, 889-894.

Favus, M. J. (1991). Intestinal Ca, Mg and P transport. In: Coe, F.L. and Favus, M.J. (Ed.) *Disorders of bone and mineral metabolism.* pp. 1-50. New York: Raven Press.

Favus, M. J., Tanbe, V., Ambrosic, K. A. and Nellans, H. N. (1989). Effect of $1,25(OH)_2D_3$ on enterocyte basolateral membrane Calcium transport in rats. *American Journal of Physiology*. **256**, G613-G617.

Favus, M. J., Tanbe, V. (1992). The use of pharmacologic agents to study mechanisms of intestinal calcium transport. ***Journal of Nutrition.*** **122.** 683-686.

Feher, J. J. (1983). Facilitated calcium diffusion by intestinal calcium binding protein. *American Journal of Physiology.* **244,** C303-C307.

Ferraris, R. P. (1994). Regulation of intestinal nutrient transport. In: Johnson, L. R.

(ed.) *Physiology of Gastrointestinal Tract*, pp 1821-1844. New York: Raven Press.

Forstner, G. G., Sabesin, S. M. and Isselbacher, K. J. (1968). Rat intestinal microvillus membranes. *Biochemical journal.* **106**, 381-390.

Frank, F. R. and Burger, R. E. (1965). The effect of carbon dioxide inhalation and sodium bicarbonate ingestion on egg shell deposition. *Poultry Science.* **44**, 1604-1610.

Franke, A. A., Custer, L. J., Cerna, C. M. and Narala, K. K. (1994). Quantitation of phytoestrogen in legumes by HPLC. *Journal of Agricultural and Food Chemistry.* **42**, 1905-1913.

Freedman, R. A., Weiser, M. M. and Isselbacher, K. J. (1977). Calcium translocation by Golgi and lateral-basal membrane vesicles from rat intestine: Decrease in vitamin D-deficient rats. *Proceedings National Academy of Science (USA)* **256**, 9932-9936.

Fuller, M. F. (1991). *In vitro digestion for pigs and poultry.* p. 209. Aberdeen, Scotland: C.A.B International.

Fullmer, C. S. (1992). Intestinal calcium absorption. *Journal of Nutrition.* **122**, 644-650.

Ghijsen, W. E. J. M. (1983). Kinetic properties of Na^{+}/ Ca^{2+} exchange in basolateral plasma membrane of rat small intestine. *Biochemica Biophysica Act.* **730**, 85-94.

Ghijsen, W. E. J. M., Dejong, M. D. and Van Os, C. H. (1980). Dissociation between Ca^{2+}-ATPase and alkaline phosphatase activities in plasma membranes of rat duodenum. *Biochimica et Biophysica Acta*, **599**: 538-551.

Ghijsen, W. E. J. M. and Van Os, C. H. (1982). 1-alpha, 25-dihydroxvitamin D_3 regulates ATP-dependent calcium transport in basolateral plasma membrane of rat enterocytes. *Bichemica Biophysica Acta.* **689**, 170-172.

Ghijsen, W. E. J. M., Van Os, C. H., Heizmann, C.W. and Murer, H. (1986). regulation of duodenal Ca^{2+} pump by calmoduline and vitamin D-dependent Ca^{2+} binding protein. *American Journal of Physiology.* **251-G**, G223-G229.

Ghishan, F. K., Leonard, D. and Pietsch, J. (1988). Calcium transport by plasma membranes of enterocytes during development: role of $1,25(OH)_2D_3$ vitamin D_3.. *Pediatric Research.* **24**, 338-341.

Gilbert, A. B. (1983). Calcium and reproductive function in the hens. *Proceeding of Nutrition Society.* **42,** *195-212.*

Giminigar, P. (1961). The calcium and phosphorous requirement of laying hens as determined by balance techniques. *Poultry and Livestock Comment.* **18**, 3-12.

Grunder, A. A. and Tsang, C. P. W. (1984). Effect of vitamin D_3 deficiency on adenosine triphosphate activity of jejunum from White Leghorn hens. *Poultry Science*. **63**, 1073-1075.

Gunder, A. A., Guyer, R. B., Buss, E. G. and Clagett, C. O. (1978). Calcium-binding in serum quantitative differences between thick and thin shell lines. *Poultry Science*. **57**, 1139-1140.

Gunder, A. A., Guyer, R. B., Buss, E. G. and Clagett, C. O. (1980). Effect of estradiol on calcium and calcium -binding in serum of thick and thin-shell lines of chickens. *Poultry Science*. **59**, 2776-2781.

Gunder, A. A., Hollands, K. G. and Tsang, C. P. W. (1981). Relationship between plasma estrogen levels and blood calcium or shell quality. *Poultry Science*. **60**, 1661-1662.

Guyer, R. B., Gunder, A. A., Buss, E. G. and Clagett, C. O. (1980). Calcium-binding proteins in serum of chickens: vitellogenin and albumin. *Poultry Science*. **59**, 874-879.

Hamaidan, F. R., Donowitz, M., Weiland, G. A. and Sharp, G. W. G. (1989). Two calcium channels in basolateral membrane of rabbit ileal epithelial cells. *American Journal of Physiology*. **257**, G86-G93.

Hamilton, R. M. and Thompson, B. K. (1980). Effect of sodium plus potassium to chloride ratio in practical-type diets on blood gas levels in three strains of white leghorn hens and relationship between acid-base balance and egg shell strength. *Poultry Science*. **59**, 1294-1303.

Harding, S. (1995). *Milk quality*. **25**, pp. 1-10. UK: Academic & professional.

Harms, R. H. (1982). The influence of nutrition on egg shell quality. part III: electrolyte balance. *Feedstuffs*. **54**, 25-28.

Harms, R. H., Rossi, A. F. and Sloan, D. R. (1990). A method for estimating shell weight and correcting specific gravity for egg weight in eggshell quality studies. *Poultry Science*, **69**: 48-52.

Harms,S. V. and Wright, E. M. (1980). Some characteristics of (sodium, potassium)-ATPase from rat intestinal basal lateral. membrane. *Journal of Membrane Biology*. **53,** 119-128.

Hughes, B. O. (1972). A circadian rhythm of calcium intake in the domestic fowl. *British Poultry Science*. **13**, 485-493.

Hughes, R. J. (1989). Influence of dietary electrolytes on blood acid-base balance in relation to formation of egg shells in the domestic hens. p.1-50. University of Adelaide.

Hunton, P. (1987). Laboratory evaluation of egg quality. In: Wells, R.G. and Belyavin, C.G. (Ed.) *Egg quality current problems and recent advance*, pp. 87-102. Butlerworths.

Hunton, P. (1995). *Poultry production.* p. 598. Ontario Canada: Elsevier.

Hunziker, W. and Bendik, I. (1996). Calbindin D-28K. In: Celio, M.R. (Ed.) *Guidebook to the calcium-binding proteins.* pp. 23-25. New York: Oxford University Press.

Hurwitz, S. (1964). Calcium metabolism of pullets at onset of egg production, as influence by dietary calcium level. Poultry Science. **43**, 1462-1472.

Hurwitz, S. (1989). Calcium homeostasis in birds. *Vitamins and hormones*. **45**, 173-221.

Hurwitz, S. and Bar, A. (1965). Absorption of calcium and phosphorus along the gastrointestinal tract of laying fowl as influenced by dietary calcium and egg shell formation. *Journal of Nutrition*. **86**, 433-437.

Hurwitz, S. and Bar, A. (1969). Calcium reserves in bones of laying hens: their presence and utilization. *Poultry Science*. **48**, 1391-1396.

Hurwitz, S. and Bar, S. (1971). The effect of pre-laying minerals nutrition on the development, performance and mineral metabolism of pullets. *Poultry Science*. **50**, 1044-1055

Hurwitz, S., Bar, A. and Cohen, I. (1973). Regulation of calcium absorption by fowl intestine. *American Journal of Physiology*. **225**, 150-154.

Hurwitz, S. and Griminger, P. (1961). Partition of calcium and phosphorus excretion in the laying hen. *Nature*. **180**, 759-760.

Jackson, M. E., Hellwig, H. M. and Waldroup, P. W. (1987). Shell quality: potential for improvement by dietary means relationship with egg size. *Poultry Science*. **66**, 1702-1713.

Jande, S. S. and Brewer, L. M. (1974). Effect of vitamin D_3 on duodenal absorptive cells of chicks, an electron microscopic study. *Arctic Anthropology Entwicklungsgesh*. **144**, 249-265.

Jande, S. S., Tolnai, S. and Lawson, D. E. M. (1981). Immunohistochemical localization of vitamin D-dependent calcium-binding protein in duodenum, kidney uterus and cerebellum of chickens. *Histochemistry*. **71**, 99-116.

Johnson, A. L. (1986). Reproduction in the female. In: Sturkie, P.D. (Ed.) *Avian physiology*. pp. 403-431. Springer-Verlag]

Karbach, U. (1992). Paracellular calcium transport across the small intestine. *Journal of Nutrition*. **122**, 672-677.

Kiyama, H., Wu, J. C. Y., Smith, M. W., Lawson, E. M. D. and Emson, P. C. (1991). Developmental control over vitamin-D-induced calbindin gene expression during early differentiation of chicken jejunal enterocytes. *Differentiation*. **46**, 69-75.

Klandorf, H., Blauwiekel, R., Qin, X. and Russell, R.W. (1998). Characterization of sustained-release estrogen implant on oviduct development and plasma Ca concentrations in broiler breeder chicks: Modulation by feed restriction and thyroid state. *General and Comparative Endocrinology*. **86**, 469-482.

Knight, D. C. and Elden, J. A. (1996). A review of the clinical effects of phytoestrogen. *Obstetrics and Gynecology*. **87**, 897-904.

Kon, O. L., Webster, R. A. and Spelsberg, T. C. (1980). Isolation and characterization of the estrogen receptor in hen oviduct: evidence for two molecular species. *Endocrinology*. **107**, 1182-1191.

Korach, K. S., Couse, J. F., Curtis, S. W., Washburn, T. F., Lindzey, J., Kimbro, K. S., Eddy, E. M., Migliaccio, S., Snedeker, S. M., Lubahn, D. B., Schomerg, D. W. and Smith, E. P. (1996). Estrogen receptor gene disruption: Molecular characterization and experimental and clinical phenotype. *Recent progress in hormone research*. **51**, 159-186.

Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J. M. and Chambon, P. (1986). The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. *The EMBO Journal* **5**, 891-897.

Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K. and Okubo, K. (1991). Malonyl isoflavone glycosides in soybean seeds (glycine max merrill). *Agricultural and Biological chemistry*. **55**, 2227-2233.

Kumar, V., Green, S., Stack, G., Berry, M., Jin, JR. and Chambon, P. (1987). Functional domains of the human estrogen receptor. *Cell*. **59**: 941-951.

Kusuhara, S. and Ohashi, T. (1991). Immunohistochemical detection of estrogen receptors in the magnum and shell gland of the chicken oviduct. *Japan Poultry Science*. **28**: 328-334.

Lane, S. M. and Lawson, D. E. M. (1978). Differentiation of the change in alkaline phosphatase from calcium ion activated adenosine triphosphatase activities associated with increased calcium absorption in chick intestine. *Biochemical Journal*. **174**, 1067-1070.

Lawson, D. E. M. (1978). *Vitamin D*. pp. 1-200. London: Academic Press.

Li, Z. Z., Bruke, W. H. and Washburn, K. W. (1986). Influence of ambient temperature and exogenous estradiol on egg production , shell strength, egg weight, plasma calcium and plasma estradiol levels of hens. *Poultry Science*. **65**, 178-179.

Lien, R. J. and Cain, J. R. (1987). Effect of dietary and parenteral estrogen and Bobwhite reproduction. *Poultry Science*. **66**, 154-161.

Lien, R. J., Cain, J. R. and Forrest, D. W. (1985). The influence of exogenous estradiol on Bobwhite Quail (Colinus Virginianus) reproductive system. *Comparative Biochemistry and physiology*. **80A**, 433-436.

McDonald. P., Edwards, R. A., Greenhalgh, J. F. D. and Morgan, C. A. (1995). *Animal nutrition*. P.543, Fourth edition New York: Longman Scientific & Technical.

Mannion, P. F. and Reichmann, K. G. (1984). *Biochemical and nutritional aspect of shell quality in the domestic hen with particular reference to calcium.* Queenisland: Animal Research Institute.

March, B. E. and Amin, M. (1981). Dietary limestone versus extra-dietary oyster shell as calcium supplements to different layer diets. *Poultry Science* **60**, 591-597.

Martin, P. M., Horwitz, K.B., Ryan, D. S. and McGuire, W. L. (1978). Phytoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinology*. **103**, 1860-1867.

Martz, A., Forte, L. R. and Langeluttig, S. (1985). Renal cAMP and 1,25 (OH)2 D_3 synthesis in estrogen-treated chick embryo and hens. *American Journal of Physiology*. **249**, E626-E633.

Matsumato, T., Fontaine, O. and Rasmussen, H. (1981). Effect of 1,25-dihydroxyvitamin D_3 on phospholipid metabolism in chick duodenal mucosa cell. *Journal of Biological Chemistry*. **256**, 3354-3360.

Matteson, D. R. and Armstrong, C. M. (1986). Properties of two calcium channel in clonal pituitary cells. *Journal of General Physiology*. **87**, 161-182.

Mcsheehy, P. M. and Chambers, T. J. (1986). Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. *Endocrinology*. **118**, 824-828

Miksicek, R.J. (1993). Commonly occurring plant flavonoids have estrogenic activity. *Molecular Pharmacology*. **44**, 37-43.

Miller, P. C. and Sunde, M. L. (1975). The effect of various particle size of oyster shell and limestone on performance of laying leghorn pullets. *Poultry Science*. **54**, 1422-1433.

Moncrief, N. D., Kretsinger, R. H. and Goodman, M. (1990). Evolution of EF-hand calcium-modulated protein. I. relationship based on amino acid sequences. *Journal of Molecular Evaluation*. **30**, 522-562.

Mongin, P. (1968). Role of acid-base balance in the physiology of egg shell formation. *Would's Poultry Science Journal.* **24**, 200-230.

Mongin, P. and Sauveur, B. (1974). Voluntary food and calcium intake by the laying hens. *British Poultry Science.* **15**, 349-356.

Muller, W. J., Brubarker, R. L. and Caplan, M. D. (1969). Egg shell formation and bone reabsorption in laying hens. *Federation Proceeding.* **28**, 1851-1856.

Mundy, G. R. (1990). Calcium homeostasis-role of the gut, kidney and bone. In: Mundy, G.R. (Ed.) *Calcium homeostasis.* pp. 1-29. Martin, Dunitz, UK

Nancy, K., Reuss, W. L. and Lemis, S. A. (1996). *Epithelial transport a guide to methods and experimental analysis.* London: Chapman.

Narushin, V. G. (1997). Non-destructive measurements of egg parameters and quality characteristics. *World's Poultry Science Journal.* **53**, 141-153.

National Research Councel (1994). *Nutrient requirements of poultry.* p. 155. 9th edn. Washington: National Academic Press.

Nemere, I. (1990). Intestinal calcium transport: Vesicular carriers, noncytoplasmic calbindin D_{28k} and non-nuclear effects of 1,25-dihydroxyvitamin D_3. In: Panus, D. and Bonner, F. (Eds.) *Calcium Transport and Intracellular Calcium Homeostasis.* pp. 233-240. New York: Springer Verlag]

Nemere, I. and Norman, A. W. (1991). Transport of calcium. In *The Gastrointestinal System (Handbook of Physiology)* (Edited by M. Fielo, & R. A. Frizzell), pp. 337-360. American Physiological Society.

Nemere, L., Yoshimoto, Y. and Norman, A. W. (1984). Calcium transport in perfused duodena from normal chick: Enhance within fourteen minutes of exposure to 1,25-dihydroxvitamin D_3. *Endocrinology.* **115**, 1476-1483.

Niggli, V., Penniston, J. T. and Carafoli, E. (1979). Effect of the Ca^{2+}-Mg^{2+} ATPase from human erythrocyte membranes using a calmodulin affinity column. *Journal of Biological Chemistry.* **259**, 9955-9958.

Nilius, B., Lansman, B. J. H. P. and Tsien, R. W. (1985). A novel type of cardiac calcium channel in ventricular cells. *Nature New Physiology Science.* **316**, 443-446.

Njoku, P. C. and Nwazota, A. O. U. (1989). Effect of dietary inclusion of ascorbic acid and palm oil on performance of laying hens in hot tropical environment. *British Poultry Science.* **30**, 831-840.

Noble, R. C., Lonsdale, F., Connor, K. and Brown, D. (1986). Change in the lipid metabolism of the chick embryo with parental age. *Poultry Science.* **65**, 409-416.

Nyholm, N. E. I. (1981). Evidence of involvement of aluminum in causation of defective formation of eggshell and impaired breeding in wild passerine birds. *Environmental Research.* **26**, 363-371.

Nys, Y., Baker, K. and Lawson, D. E. M. (1992). Estrogen and a calcium flux dependent factor modulate the calbindin gene expression in the uterus of laying hens. *General and Comparative Endocrinology.* **87**, 87-94.

Nys, Y. and Laage, X. D. (1984). Effect of suppression of eggshell calcification and of $1,25(OH)_2D_3$ on Mg^{2+}, Ca^{2+} and $Mg^{2+}HCO_3$ ATPase, alkaline phosphatase, carbonic anhydrase and CaBP levels-II. The laying hen intestine. *Comparative Biochemistry and Physiology.* **78A**, 839-844.

Pansu, D., Bellaton, C., Roche, C. and Bronner, F. (1989). Theophylline inhibits transcellular Ca transport in intestine and Ca by CaBP. *American Journal of Physiology.* **257**, G935-G943.

Pansu, D., Chapuy, M. C., Milani, M. and Bellaton, C. (1976). Transepithelial calcium transport enhanced by xylose and glucose in the rat jejunal lighted loop. *Calcified Tissue Research.* **21**, 45-52.

Panus, D., Bellaton, C. and Bronza, F. (1983). Development change in the duodenal calcium transport in the rat. *American Journal of Physiology.* **244**, G20-G26.

Pappenheimer, J. R. (1987). Physiological regulation of transepithelial impedance in the intestinal mucosa of rats and hamster. *Journal of Membrane Biology.* **100**, 137-148.

Parker, M. G. (1995). Structure and function of estrogen receptor. *Vitamins and Hormones.* **51**, 267-287.

Patton, M. B. and Sutton, T. S. (1952). The utilization of calcium from lactate, gluconate, sulfate and carbonate salts by young college woman. *Journal of Nutrition.* **48**, 443-452.

Petersen, C. F. (1965). Factors influencing egg shell quality. *World's Poultry Science Journal.* **21**, 110-138.

Petterson, H. and Kiessling, K. H. (1984). Liquid chromatographic determination of plant estrogens coumestrol and isoflavones in animal feed. *Association of Official Analytical Chemists Journal.* **67**, 503-506.

Picotto, G., Massheimer, V. and Boland, R. (1996). Acute stimulation of intestinal cell calcium influx induced by 17B-estradiol via the cAMP messenger system. *Molecular and Cellular Endocrinology.* **119**, 129-134.

Pike, J. W., Spanos, E., Colston, K. W., Macintrye, I. and Haussler, M.R. (1978). influence of estrogen on renal vitamin D hydroxylases and serum 1a,25-(OH)2D3 in chicks. *American Journal of Physiology.* **235**, E338-E343.

Pond, W. G., Church, D. C. and Pond, K. R. (1995). *Basic animal nutrition and feeding*. p. 615, Fourth edition, New York: John Willy & Sons.

Pourreza, J., Nili, N. and Edriss, M. (1994). Relationship of plasma calcium and phosphorus to the shell quality of laying hens receiving saline drinking water. *British Poultry Science.* **35**, 755-762.

Qin, X. and Klandrof, H. (1993). Effect of estrogen in relation to dietary vitamin D_3 and calcium on activity of intestinal alkaline phosphatase and Ca-ATPase in immature chicks. *General and Comparative Endocrinology,* **90**, 318-327.

Qin, X. and Klandrof, H. (1995). Effect of estrogen on egg production, shell quality and calcium metabolism in molted hens. *Comparative Biochemical and Physiological.* **110**, 55-59.

Qin, X., Klandrof, H., Porter, D. W., Holt, S. B. and Martin, W. G. (1993). Estrogen enhancement of Ca-, Mg-, and Ca-Mg stimulated adenosine triphosphatase activity in the chick shell gland. *General and Comparative Endocrinology.* **89**, 4-10.

Quigley, J. P. and Gotterer, G. S. (1969). Distribution of (Na^+-K^+)-stimulated ATPase activity in rat intestinal mucosa. *Biochemica et Biophysica Acta.* **173**, 456-468.

Rasmussen, H., Matsumoto, T., Fontaine, O. and Goodman, D. B. P. (1982). Role of changes in membrane lipid structure in action of 1, 25-dihydroxvitamin D_3. *Federation Proceeding.* **41**, 72-77.

Reinli, K. and Block, G. (1996). Phytoestrogen content of foods-A compendium of literature values. *Nutrition and Cancer.* **26**, 123-148.

Roberts, J. R. and Brackpool, C. (1994). The ultrastructure of avian egg shell. *Poultry Science.* **5**, 245-271.

Roberts, J. R. and Brackpool, C. (1995). *Egg shell ulterastructure and the assessment of egg shell quality.* Armidale: University of New England.

Robey, W. W., Van-Krey, H. P., Cherry, J. A., Siegel, P. B. and Abliss, B. A. (1988). Regenerative capacity of avian adipose tissue in response to partial lipectomy and estrogen administration. *Archiv Fuer Gefluegelkunde.* **52**, 234-239.

Roche, C., Bellaton, C., Pansu, D., Miller, A. and Bronner, F. (1986). Localisation of vitamin D-dependent active Ca^{++} transport in rat duodenum and relation to CaBP. *American Journal of Physiology.* **251**, G314-G320.

Ross, T. K., Darwish, H. M. and Deluca, H. F. (1994). Molecular biology of vitamin D action. In: Wack, G. L. (ed.) *Vitamins and hormones advance in research and application steroids.* **49**: 281-326.

Ronald, D. A., Putman, C.E. and Hilburn, R. L. (1978). The relationship of age with ability of hens to maintain eggshell calcification when stressed with inadequate dietary calcium. *Poultry Science*. **57**, 1616-1620.

Rubinoff, M. J. and Nellans, H. N. (1985). Active calcium sequestration by intestinal microsomes stimulation by increased calcium load. *Journal of Biological Chemistry*. **260**: 7824-7828.

Ruschkowski, R. S. and Hart, L. E. (1992). Ionic and endocrine characteristics of reproductive failure in calcium-deficient and vitamin D-deficient laying hens. *Poultry Science* 1722-1732.

Sallis, J. D. (1962). Calcium metabolism in the chicken, with reference to vitamin D_3 University of Adelaide. pp.1-300.

Sauvenur, B. and Picard, M. (1987). Environmental effect on egg quality. In: Well, R. G. and Belyavin, C.G. (Ed.) *Egg quality current problems and recent advance*. pp. 219-234. Butlerworths.

Schachter, D. and Rosen, S. M. (1959). Active transport Ca^{45} by the small intestine and its dependence on vitamin D. *American Journal of Physiology*. **196**, 357-362.

Schiffi, H. and Binswager, U. (1980). Calcium ATPase and intestinal calcium transport in ureic rats. *American Journal of Physiology*. **238**, G424-G428.

Schwabe, J. W. R., Chapman, L., Finch, J. T. and Rhodes, D. (1993). The crystal structure of the estrogen receptor DNA-binding domain to DNA: how receptors discriminate between their response elements. *Cell*. **75**, 567-578.

Scott, M. L., Nesheim, M. C. and Young, R. J. (1982) Essential inorganic elements. In: Anonymous *Nutrition of chicken*. pp. 277-382. New York: Humphrey Press.

Scott, M. L. and Zeigler, T. R. (1963). Evidence for natural chalets which aid in the utilisation of zinc by chicks. *Journal of Agriculture*. **11**, 123-128.

Sebastian, S., Touchburn, S. P. and Chavez, E. R. (1998). Implications of phytic acid and supplemental microbial phytase in poultry nutrition: a roe. *World's Poultry Science Journal,* **54**: 27-47.

Setchell, K. D. R. and Welsh, M. B. (1987). High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical and thermospray mass spectrometric detection. *Journal of Chromatography*. **386**, 315-323.

Shimura, F. and Wasserman, R. H. (1984). Membrane-associate vitamin D-induced calcium binding protein (CaBP): Quantification by radio-immunoassay and evidence for specific CaBP-binding protein in purified

intestinal brush border. *Endocrinology*. **115**, 1-9.

Shiwki, T., Takahashi, N., Kawate, N. , and Suda, T. (1982). The possible role of calcium binding protein induced by 1,25-dihydroxyvitamin D_3 in the intestinal calcium transport mechanism. *Endocrinology*. **111**, 1546-1551.

Sim, J. S. and Nakai, S. (1994). *Egg uses and processing technologies new devilment.* p.436. UK: CAB International.

*Simikiss, K. (1961).** Calcium metabolism and avian reproduction. *Biology Review* **36**, 321-367.

Simkiss, K. and Tayler, T. G. (1971). Shell formation. In: Bell, D. J. and Freeman, B.M. (Ed.) *Physiology and biochemistry of the domestic fowl.* pp. 1331-1343. London: Academic Press.

Simmen, R. C. M., Means, A. R. and Clark, J. H. (1984). Estrogen modulation of nuclear matrix-associated steroid hormone binding. *Endocrinology*. **115**, 1197-1202.

Smith, M. W. (1990). Peptide and amino acid absorption in the small intestine. In: Mellinger, J. (Ed.) *Animal nutrition and transport processes.* pp. 146-155. Paris: Karger.

Smith, M. W and Peacock, M. A. (1989). Comparative aspects of microvillus development in avian and mammalian enterocytes. *Comparative Biochemistry and Physiology*. **93A,** 617-622.

Smith, M. W., Paterson, J. Y. F. and Peacock, M. A. (1984). A comprehensive description of brush border membrane development applying to enterocytes taken from a wide variety of mammalian species. *Comparative Biochemistry and Physiology*. **77A**, 655-662.

Smith, U. and Deluca, H. F. (1993). The necessity for calcium for increased renal vitamin D receptor in response to 1, 25-dihydroxyvitamin D. *Biochemica et Biophysica Acta*. **1176**, 321-326.

Solomon, S. E. (1991). *Egg and eggshell quality.* p. 149.London, England: Wolfe Publishing LTD.

Sommerville, B. A., Scanes, C. G., Swaminathan, R., Care, A. D., Harvey, S. and Chawick, A. (1989). Effect of estrogen on calcium homeostasis and pituitary hormones in the growing chick. *General and Comparative Endocrinology*. **76**, 261-266.

Spencer, R., Charman, M., Wilson, P. W. and Lawson, D. E. M. (1978). The relationship between vitamin D-stimulated calcium transport and intestinal calcium-binding protein in the chicken. *Biochemical Journal*. **170**, 93-101.

Standelman, W. J. and Cotterill, O. J. (1995). *Egg science and technology.* p. 591. USA: Harvard Press, Inc.

Sutherland, R. L. and Baulieu, E.E. (1976). Quantitative estimates of cytoplasmic and nuclear oestrogen receptor in chick oviduct. *European Journal Biochemistry.* **70**, 531-541.

Suzuki, S., and Kono, T. (1980). Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intercellular storage site. *Proceeding National Academy Science(USA)* **77**, 2542-2545.

Takahashi, N., Shinki, T., Kawate, K., Nishii, Y. and Suda, T. (1982). Distribution of ornithine decarboxylase activity induced by 1,25-dihydroxyvitamin D_3 in chick villus mucosa. *Endocrinology.* **111**, 1539-1545.

Takito, J., Shinki, T., Sasaki, T. and Suda, T. (1990). Calcium uptake by brush border and basolateral membrane vesicles in chick duodenum. *American Journal of Physiology.* **258**, G16-G23.

Taylor, T. G., Simikiss, K and Stringer, D. A. (1971). The skeleton-its structure and metabolism. In: Dell, D. J. and Freeman, B. M. (Ed.) *Physiology and Biochemistry of the Domestic fowl.* pp.621-640. London: Academic Press.

Taylor, R. D., Nolan, J. V. and Jones, G. P. D. (1995). Some more consequences of the intermittent feeding of particulate calcium to laying hens. In: Jawes, B. R and Nolan J. V (Ed.) *Recent advances in animal nutrition in Australia.* pp. 62-68. Armidale: University of New England

Thomasset, M., Malla, A., Parker, O. and Demaille, J. G. (1981). Intestinal calmodulin and calcium binding protein differ in their distribution and in the effect of vitamin D steroids on their concentration. *Federation European Biochemical Societies.* **127**, 13-16.

Tsang, C. P. W. (1988). Effects of cholecalciferol or calcium deficiency on estrogen metabolism in the laying hen. *British Poultry Science.* **29**, 753-759.

Tullet, S. G. (1987). Egg shell formation and quality. In: Well, R. G. and Belyavin, C. G. (Ed.) *Egg quality current problems and recent advance.* pp. 123-146. Butlerworths.

Turner, C. W. (1948). Feeding estrogen (Dianisylhexene) to laying hens. *Poultry Science.* **27**, 593-600.

Uni, Z., Noy, Y. and Sklan, D. (1995). Development of the small intestine in heavy and light strain chicks before and after hatching. *British Poultry Science* **36,** 63-71.

US.Bureau of the Census, International Data, Base accessed on 28, July, (1998). *.http:/www.census.gov/ipc/www/idbnew ptml.*

Ussing, H. H. and Johansen, B. (1969). Anomalous transport of sucrose and urea in toad skin. *Nephron.* **6**, 317-328.

Van Corven, E. J. I., De Jong, M.D. and Van Os, C.H. (1986). Enterocyte isolation procedure specifically effects ATP-dependent Ca^{2} -transport in small intestinal plasma membrane. *Cell Calcium.* **7**, 89-99.

Van Corven, E. J. M., Roche, C. and Van Os. C. H. (1985). Distribution Ca^{2+-} ATP-dependent Ca^{2+}transport, calmodulin and vitamin D-dependent Ca^{2+}-binding protein along the villus-crypt axis in rat duodenum. *Biochimica et Biophsica Acta.* **820**, 274-282.

Van Os, C. H. (1987). Transcellular calcium transport in intestine and renal epithelial cells. *Biochemica Biophysica Acta.* **906**, 195-222.

Vaughan, O. W. and Filer, L. J. (1960). The enhancing action of certain carbohydrates on the intestinal absorption of calcium in the rat. *Journal of Nutrition.* **71**, 10-14.

Wall, PM. L. and Samman, S. (1997). Flavonoids-dirtary perspective and health benefits. *Proceeding of the Nutrition Society of Australia.* **21**, 106-114

Walter, J. R. and Weiser, M. M. (1987). Calcium transport by rat duodenal villus and crypt basolateral membranes. *American Journal of Physiology.* **252**, 10-14.

Wang, G., Kuan, S. S., Francis, O. J., Ware, G. M. and Carman, A. S. (1990). A simplified HPLC method for the determination of phytoestrogen in soybean and its processed products. *Journal of Agricultural and Food Chemistry.* **38**, 185-190.

Wanner, R. R. and Coleman, J. R. (1975). Electron probe analysis of calcium transport by small intestine. *Journal of Cell Biology.* **64**, 54-74.

Wasserman, R. H. (1981). Intestinal absorption of calcium and phosphorus. *Federation Proceeding.* **40**, 68-72.

Wasserman, R. H., Chandler, J. S., Mayer, S. A., Smith, C. A., Brindark, M. E., Fullmer, C. S., Penniston, J. T. and Kumar, R. (1992). Intestine calcium transport and calcium extrusion processes at the basolateral membrane. *Journal of Nutrition.* **122**, 662-671.

Wasserman, R. H., Comar, C. L. and Nold, M. M. (1956). The influence of amino acids and other organic compound on the gastrointestinal absorption of calcium 45 and strontium 89 in rat. *Journal of Nutrition.* **59**, 371-383.

Wasserman, R. H., Fullmer, C. S. and Taylor, A. N. (1978). The Vitamin D-dependent calcium-binding protein. In: Lawson, D. E. M. (Ed.) *Vitamin D.*

pp. 133-165. London: Academic Press.

Watanabe, E., Kobyashi, S., Terashima, Y. and Itoh, H. (1987). Adenosine triphosphatase in the uterus and duodenum of chicken hens during eggshell formation. *Poultry Science*. **68**, 564-568.

Weiser, M. M., Bloor, J. H., Dasmahapatra, A., Freeman, R. A. and Maclaughlin, J. A. (1981). Vitamin D-dependent rat intestinal Ca^{2+} transport, calcium uptake by golgi membrane and nuclear events. In: Bronner, F. and Peterlik, M. (Ed.) *Calcium and Phosphate Transport across Biomembrane*. pp. 264-273.

Welshone, W. V., Rottinghaus, G. E., Nonneman, D. J., Dolan-Timpe, M. and Ross, P.F. (1990). A sensitive bioassay for detection of dietary estrogen in animal feeds. *Journal of Veterinary Diagnostic Investigation*. **2**, 268-273.

Wilson, P. W. and Lawson, D. E. M. (1980). Calcium binding activity by chick intestinal brush border membrane vesicles. *Pfugers Archive European Journal Physiology*. **389**, 69-74.

Wu, J. C. Y., Smith, M. W., Mitchell, M. A., Peacock, M. A., Turvey, A. and Keable, S. J. (1993). Enterocyte expression of calbindin, calbindin mRNA and calcium transport increases in jejunal tissue during onset of egg production in the fowl (Gallus Domesticus). *Comparative Biochemistry and Physiology*. **106A**, 263-269.

Wu, J. C. Y., Smith, M. W., Turvey, A., Keable, S. J. and Colston, K. W. (1994). Differential regulation of vitamin D receptor and intestinal calcium transport occurring during sexual maturation in the fowl (Galus Domesticus). *Comparative Biochemistry and Physiology*. **109**, 713-720.

Yoshimura, Y., Okamoto, T and Tamura, T (1995). change in localization immunoreactive estrogen receptor during follicular development in hens. *General and Comparative Endocrinology*. **100**, 368-374.

Yu, B. and Chiou, W. S. (1996). Effects of crude fibre level in the diet on the intestinal morphology of growing rabbits. *Laboratory Animals*. **30**, 143-148.

APPENDIX

PUBLICATIONS

Saki, A. A. & Tivey, D. R. (1997). Immunohistochemical detection of Calbindin D28K and oestrogen receptor in the small intestine of pre- and post-lay hens. In D. Balnave et al. (eds.) *The Australian Poultry Science Symposium.* (p. 240). The Poultry Research Foundation, University of Sydney.

NOTE:

This publication is included in the print copy
of the thesis held in the University of Adelaide Library.

Saki, A. A. & Tivey, D. R. (1997). Egg characteristics of Leghorn and Tegel pullets in response to interactin between estrogen and calcium. In *Proceedings of the Nutrition Society of Australia.* (p. 149).

NOTE:

This publication is included in the print copy
of the thesis held in the University of Adelaide Library.

Saki, A. A., Dalais, F. S., Zarrinkalam, M. R. & Tivey, D. R. (1998, June). Egg and egg shell characteristics of Leghorn pullets in response to soybean and exogenous estrogen. In *Proceedings 6th Asian Pacific Poultry Congress.* (p. 390-391). Japan Poultry Science Association, Nagoya, Japan.

NOTE:

This publication is included in the print copy
of the thesis held in the University of Adelaide Library.