GROWTH, CARCASS AND DIGESTIVE SYSTEM CHANGES IN HYBRID VILLAGE CHICKENS AFTER UROPYGIALECTOMY

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By

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Akar Putra is a hybrid village chicken; the cross breeding process was happened by chance when the wild jungle fowl interred Universiti Putra Malaysia (UPM) ground and mated with their Ayam Kampung (Native chicken). Nonetheless, there is a dearth of information relating to the Akar Putra chicken strain, particularly the morphology of its digestive system.

Uropygial Gland (UG) is the most prominent integument gland in the birds. It is puzzling that little is known about its morphology and function. The suggested functions of this gland can be placed into four groups: 1) feather maintenance; 2) water-proofing; 3) intraspecific communication and 4) defense against predators.

This thesis introduces a new technique to improve the production performance of the chicken through ablation of the uropygial gland which called Uropygialectomy (UP). This application will cause an upset in the poultry industry as well as will significantly contribute towards its development by increasing the economic viability obtained through an increase the chicken product. Previous method of Uropygialectomy included removing the uropygial gland completely and that usually attached by severe bleeding with hard stress exposure of the chicken. While, our modification method includes removing parts from the gland (half lobes, half isthmus and papillae). That modification was taken place in order to make UP operation safer, practical, applicable and shows a significant improvement of production performance.

Therefore, this study was carried out to address the production performance, carcass characteristics, external morphological changes, anatomical changes of digestive system, growth hormone concentration and histology of digestive system investigation of 120 Akar Putra chickens strain following the ablation of the uropygial gland. The experiment comprised five treatments, with 3 replicates for each. The treatments consisted of a control T1; Uropygialectomy was applied with T2, T3, T4 and T5 treatments at 3, 4, 5 and 6 weeks of age respectively.

The results revealed remarkable significant ($P < 0.05$) enhancing for UP treatments than a control group in all of males and females' body weight, weight gain, feed intake and feed conversion ratio measurements. Furthermore, the results indicated that UP treatments caused significantly improvement ($P < 0.05$) concerning body weight, carcass weights and dressing percentage with or without eating giblets. Additionally, significant different at level ($P < 0.05$) was observed in the traits of males' breast and back relative weight. Additionally, there was a significant effect at level ($P < 0.05$) in the females' breast relative weight trait; however, T2 surpasses other treatment groups T1, T3, T4 and T5 with relation to the most carcass traits involved in this experiment.

The external morphological comparison results between UP treatments and control at week 12 shows that the males of UP treatments had higher values in bird length, growth rate, breast diameters and lengths of neck, back, keel bone and extremities than males of the control group. Likewise, females of PU treatments surpassed females of the control group in bird length, growth rate, neck length, breast width and comb length.

The anatomical evaluations revealed that the males of UP treatments had more length ($P < 0.05$) esophagus 9.9-16.2%, proventriculus 11.1-34.4%, gizzard 26.7-220%, pancreas 0-20.4%, jejunum 4.9-26.1 and colon 18.1-60.6 than their control group counterparts. Furthermore, females of UP treatments had ($P < 0.05$) longer esophagus 6.8-22.3%, pancreas 8.3-33.3% and cecum 13-26% compared with females in control.

Histologically, surgical removing of the uropygial gland, especially at week 3 had greater ($P < 0.05$) effect on the total duodenum, jejunum and ilium wall thickness. In addition, effects ($P < 0.05$) were observed on the wall thickness of males' cecum and colon. Moreover, the wall layers of: esophagus, proventriculus, gizzard and rectum were not affected by the treatment. However, removing the uropygial gland showed significant impact ($P < 0.05$) in males' growth hormone concentration level at week 7 and ($P < 0.05$) effect at week 12 in both sexes.

In conclusion, the results of current study demonstrated that partial ablation of the uropygial gland, especially at week 3 of age had a positive effect on the production performance, carcass characteristics, external morphological measurements, anatomy and histology of digestive system organs as well as the growth hormone concentration of the Akar Putra chicken strain. These enhancement in the body performance can be justified that UP will contribute in retention the essential fatty acids in the body and avoid its attraction inside the UG, then secreted out of the body. In another hand, it will support the work of prostaglandins, which drives from Arachidonic fatty acid, resulting in the production of growth hormone. The improvement in the productive performance, carcass characteristics, phenotypic traits, as well as the anatomy and histology of the digestive system were as a consequence to increase of growth hormone secretion by the anterior pituitary gland. Moreover, the improvement in the body morphology was as a consequence to increase of the steroid hormones' levels by stopping the function of the uropygial gland in converting progesterone to testosterone.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PERUBAHAN TUMBESARAN, KARKAS DAN SISTEM PENCERNAAN DI DALAM AYAM KAMPUNG HYBRID SELEPAS UROPIGIALEKTOMI

Oleh

HASAN SAAD ABDULHUSSEIN JAWAD

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Ayam Akar Putra merupakan sejenis baka kacukan yang telah melalui proses pembiakan silang yang berlaku secara kebetulan apabila ayam hutan liar memasuki kawasan Universiti Putra Malaysia (UPM) dan mengawan dengan Ayam Kampung (ayam tempatan). Walau bagaimanapun, terdapat kekurangan maklumat berkaitan dengan ayam Akar Putra, terutamanya morfologi sistem pencernaan.

Kajian ini memperkenalkan kaedah yang baharu untuk mempertingkatkan prestasi pengeluaran melalui penyingkiran kelenjar urophagial. Kaedah ini akan memberi kesan yang ketara kepada nilai ekonomi melalui peningkatan produk ayam. Kaedah yang terdahulu, dimana kelenjar ini dibuang sepenuhnya melalui pembedahan mengakibatkan pendarahan dan tekanan yang teruk kepada ayam. Kaedah yang baharu ini adalah penyingkiran separuh dari kelenjar urophagial (separuh lobes, isthmus dan papillae) melalui pembedahan. Pengubahsuaian kaedah ini adalah untuk menghasilkan pembedahan yang lebih selamat, praktikal, boleh digunapakai dan menghasilkan perubahan yang ketara kepada prestasi pengeluaran.

Oleh itu, kajian ini dijalankan bagi mengkaji prestasi pengeluaran, ciri-ciri karkas, perubahan morfilogi luaran, perubahan anatomi sistem penghadaman, kepekatan hormon pertumbuhan dan histologi sistem penghadaman pada 120 ayam Akar Putra selepas penyingkiran sebahagian kelenjar uropygial (Uropygiallectomy). Ekperimen ini dibahagikan kepada lima kumpulan dan 3 replicate. Kumpulan ini terdiri dari; T1 untuk kawalan, T2, T3, T4 dan T5 pada minggu ke 3, 4, 5 dan 6.

Hasil kajian menunjukkan penyingkiran sebahagian dari kelenjar uropygial (PU) menunjukkan perbezaa yang ketara ($P < 0.05$) diantara kumpulan kawalan dan kumpulan eksperimen pada ayam jantan dan betina dari segi pertambahan berat badan, pengambilan makanan, kadar penukaran makanan, dan berat reletif otot dada bagi jantan dan betina. Ianya juga menunjukkan perbezaan yang ketara ($P < 0.05$) bagi berat hidup dan berat karkas dengan giblet dan juga tampa giblet. Kumpulan T2

menunjukkan kesan yang paling ketara diantara kumpulan-kumpulan eksperimen yang terlibat.

Perbandingan morfologi luaran pada minggu ke 12 menunjukkan, ayam jantan menghasilkan nilai yang lebih tinggi untuk berat dan panjang badan, kadar tumbesaran, diameter otot dada dan panjang leher, belakang dan tulang kaki daripada kumpulan kawalan, manakala ayam betina menunjukkan nilai yang lebih tinggi pada berat badan dan panjang badan, kadar tumbesaran, panjang leher dan balung serta lebar otot dada.

Penilaian anatomi menunjukkan PU pada ayam jantan menghasilkan ($P<0.05$) 9.9-16.2% esophagus lebih panjang, 11.1- 34.4% pada proventrikulus, 26.7 %-220% pada hempedal, 0-20.4% pankreas, 4.9-26.1% jujunum dan 18.1-60.6% kolon daripada kumpulan kawalan. Manakala PU pada ayam betina menunjukkan ($P<0.05$) 6.8-22.3% esophagus lebih panjang, 8.3-33.3% pankreas dan 13-26% pada sekum berbanding dengan kumpulan kawalan.

Secara histologinya, PU pada umur 3 minggu menunjukkan kesan yang ketara ($P < 0.05$) pada ketebalan duodenum, jujunum dan ilium. Walaubagaimana pun ketebalan esophagus, proventrikulus, hempedal dan rektum tidak menunjukkan perbezaan yang ketara. PU pada ayam jantan menunjukkan perbezaan yang ketara ($P < 0.05$) bagi hormon tumbesaran pada umur 7 minggu dan perbezaan yang ketara ($P < 0.05$) pada umur 12 minggu untuk ayam jantan dan betina.

Kesimpulannya, PU pada umur 3 minggu menunjukkan kesan yang positif kepada prestasi pertumbuhan, krateria karkas, ukuran morfologi luaran, anatomi dan histologi organ-organ pencernaan termasuk konsentrasi hormon tumbesaran pada ayam Akar Putra.

Penambahbaikan prestasi badan ayam ini disebabkan kerana, kaedah UP ini menyumbang kepada pengekalan asid lemak penting di dalam badan dan menghalang kepada tarikan kepada kelenjar urophagial. Ia juga membantu fungsi hormon prostaglandin yang terhasil dari asid lemak Arachidonic dalam penghasilan hormon tumbesaran. Peningkatan prestasi pengeluaran, krateria karkas, sifat-sifat fenotip termasuk anatomi dan histologi system pencernaan adalah akibat dari peningkatan penghasilan hormon tumbesaran yang dihasilkan dari kelenjar Pitutari anterior. Tambahan pula peningkatan kepada morfologi badan adalah disebabkan peningkatan paras hormon steroid seterusnya penukaran hormon progestron kepada testosteron dengan menyekat fungsi kelenjar urophagial.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

UP	Uropygialectomy
UG	Uropygial gland
PI	Production Index
EPEF	European Production Efficiency Factor
ANOVA	Analysis of variance
cm	Centimeter
d	Day
g	Gram
GIT	Gastrointestinal tract
H & E	Haematoxylin and Eosin
ml	Milliliter
NS	Normal Solution
NBF	Neutral Buffered Formalin
nm	Nanometer
PBS	Phosphate Buffered Saline
SE	Standard Error
SPSS	Statistical Package for the Social Sciences
vol	Volume
♂	Male
♀	Female
trt	Treatment
rept	Replicate
RW	Relative Weight
RL	Relative Length
GH	Growth Hormone
GHRH	Growth Hormone Releasing Hormone
IGF-I	Insulin-like Growth Hormone

CHAPTER 1

INTRODUCTION

Akar Putra is a hybrid village chicken; the cross breeding process was happened by chance when the wild jungle fowls entered Universiti Putra Malaysia grounds and mated with their chicken (ayam kampong). Akar Putra chicken has a more robust growth process than its parents because the maturation period is shorter (less 13 weeks). It can lay 120-200 eggs per year, and it has more resistance for diseases (Kasim, 2007).

Uropygial gland is the only subcutaneous gland in birds' body (Mclelland, 1990). It has many names like preen gland, based on its function in preening the bird's feather (Lucas and Stettenheim, 1972a; King and Mclelland, 1984) and oil gland depending on its oily secretion (Schultz *et al*., 2002). Furthermore, it called uropygial gland based on its position, which is on the base of the tail, dorsally between the fourth caudal vertebrae and the pygostile (Lucas and Stettenheim, 1972a,b ; Sawad, 2006a). The function of the gland is still a subject of controversy. There are many accepted functions of gland secretions like conferring water-repellent properties on the feather coat and maintaining the suppleness of it. In addition, it has proposed to be associated to pheromone production, control of plumage hygiene, thermal insulation and defense against predators because of its foul small in some birds (Jacob, 1992; Montalti *et al*., 2000; Soler *et al*., 2012; Vincze *et al*., 2013). Uropygial gland is completely absent in Struthionidae, Rheidae, Casuaridae, Dromaidae and in a few species of Columbidae and Psittacidae (Johnston, 1988). Montalti and Salibián (2000) mentioned that the oil of the uropygial gland is not important to the birds who do not have it. While Goodwin (1970) said, the uropygial gland in some of the birds is non-active. Moreover, Moyer *et al*., (2003a, b) gave an explanation that the birds that do not have uropygial gland use dusters bath to keep and clean their feather.

Modern commercial breeds of meat chicken characterized by super-fast growth and high efficiency of a food conversion ratio as a result of intense genetic selection. Wepruk and Church (2003) observed that the final body weight of broiler in 1976 was 2 kg at the age of 63 days while the same average of body weight was arrived at age 35 days in 2001. This improvement in the growth rate reflected negatively on the disease resistance and immune response of these birds, because of a negative genetic link coefficient was observed between the growth speeds and immune response (Qureshi and Havenstein, 1994). In this context, increasing in a mortality ratio in these strains of birds happened due to increasing their susceptibility to bacterial diseases and metabolic diseases. These occurred as a consequence of irregular metabolic processes, an imbalance in the acid-base balance of body fluids such as ascites disease, sudden-death syndrome (SDS) and increased skeletal disorders like leg abnormalities. It has been scientifically proven that highest rates of those pathological conditions were shown in flocks and individual rapid growth chicken at 3rd and 4th weeks of age (Robinson *et al*., 1992; Julian, 1997, 1998; Leeson and Summer, 1997; Gonzales *et al*., 1998, 2000). Based on the limitation of the problem, this research was planned to innovate a safe

technique to raise the level of poultry production performance in general and local Malaysian chicken (Akar Putra strain) particularly without using the genetic improvement methods, which have proven that it has negative impacts on birds' immunity. Furthermore, this study planned to reduce the gap in knowledge in terms of the production performance, carcass characteristics and digestive system morphology of the hybrid village chicken. Regarding on uropygialectomy (UP), earlier studies were described that application as one of the improvement methods to enhance the body performance of chicken. Previous method of UP included removing the uropygial gland completely and that usually attaches with severe bleeding which sometimes case decimation of the experimental birds. Additionally, it has been reported that high numbers of the treated birds could be dying after the operation because of cannibalism, which is sometimes difficult to overcome. The reason behind that may be the remaining blood on the feathers around the incision area after the operation, which is considered as an irritation factor for the chicken and urges them to cannibalism. Furthermore, the previous researchers recommended to apply the uropygialectomy at the earlier ages (1-6) weeks of chicken age in order to give the body sufficient time to grow up (AI-Daraji *et al*., 2006). In addition, because of their observation that the adult chicken has to be less responsive to the uropygialectomy compared than the chicken in growing stage. The UP in this study has been modified to include removing about half lobes, half isthmus and papillae at the growing stage (3-6 weeks). That modification was taken place in order to make UP operation safer, practical and applicable (quickly and accurately), which encourages to perform it on a large scale in the poultry farms.

The aim of this study is to describe the anatomical and histological changes of the digestive system and the growth patterns of the digestive organs as well as the carcass characteristics after ablation of the uropygial gland, which was at weeks 3, 4, 5 and 6 of age on T2, T3, T4 and T5 treatments respectively. In this study, we also examined the weekly production performance, production index (PI) and European production efficiency factor (EPEF), which are considered the most confidence standards to evaluate poultry farm experiments in the economic term. Furthermore, the external morphology and plasma growth hormone at weeks 7, 10 and 12 of age for males and females separately as an attempt to cover their changes in the period after the last application of uropygialectomy at week six until the end of the experiment (week 12). The hypothesis that the morphology and histology of digestive system organs as well as production performance, carcass characteristics, external morphology and plasma growth hormone are affected by UP. It is worthy to mention that the objectives of this study have been covered for males and females separately in order to be more economic importance in the poultry industry. Therefore, it is known that the new trend of the poultry industry is a single-sex breeding. For example, some companies adopt a male breeding, and others adopt breeding females, in line with the markets need and the desire of consumers. Moreover, the current study has compared each part, organ or tissue between the treatments separately without any interaction in order to provide the maximum amount of information and detail that shows which treatment was more affected by ablation of the uropygial gland. That corresponds with the data analysis new approach in the most professional poultry journals. This study was undertaken with the following objectives:

- To define the production performance of Akar Putra chicken as a new strain of village chicken and its effectiveness by UP at different ages.
- To compare the carcass characteristics between UP treatments and control group.
- To compare the external morphological parameters at 3 different ages (7, 10 and 12 weeks) between UP treatments and control group.
- To investigate the anatomical changes of the digestive system organs after ablation of the uropygial gland.
- To assay the histological changes of the digestive system organs as well as the plasma growth hormone at 3 different ages (7, 10 and 12 weeks) between UP treatments and control group.

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APPENDICES

CHAPTER 4

Appendix 4.1

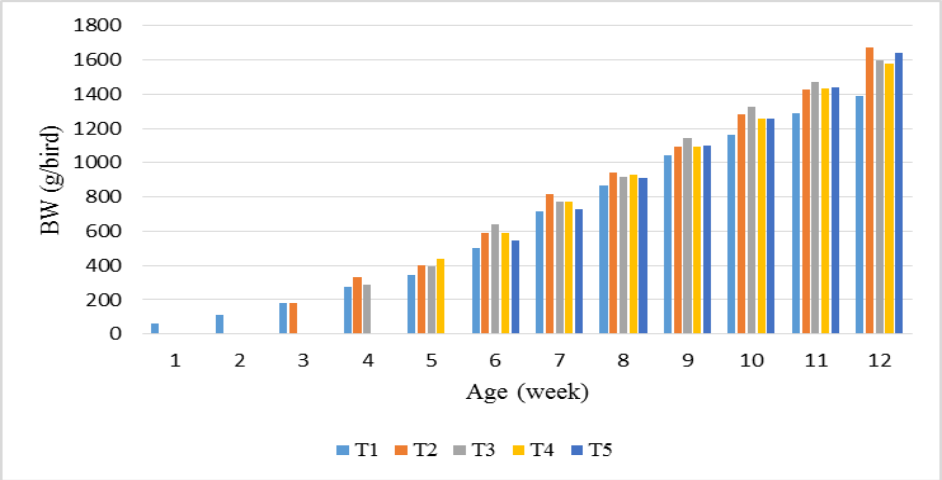


Figure 4.1: Graph of males' body weight effectiveness according to the proceeding time of uropygialectomy.

Appendix 4.2

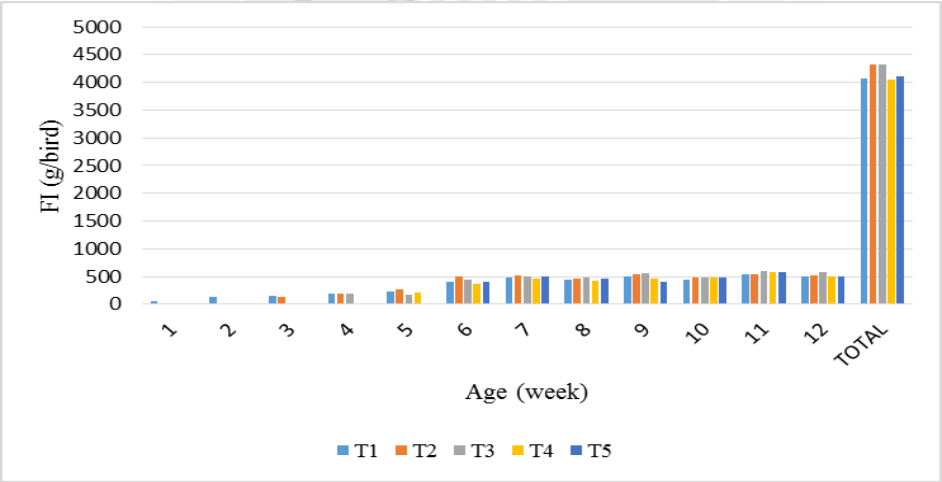


Figure 4.2: Graph of males' feed intake effectiveness according to the proceeding time of uropygialectomy.

Appendix 4.3

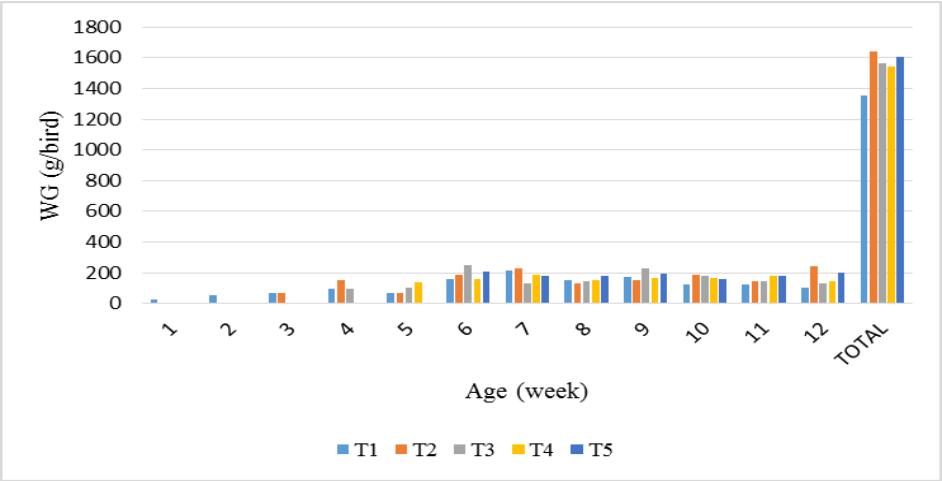


Figure 4.3: Graph of males' weight gain effectiveness according to the proceeding time of uropygialectomy.

Appendix 4.4

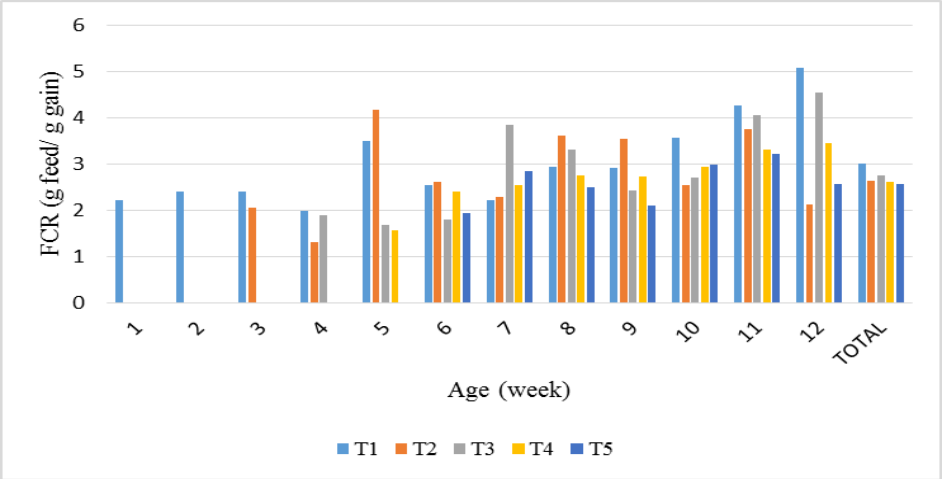


Figure 4.4: Graph of males' feed conversion ratio effectiveness according to the proceeding time of uropygialectomy.

Appendix 4.5

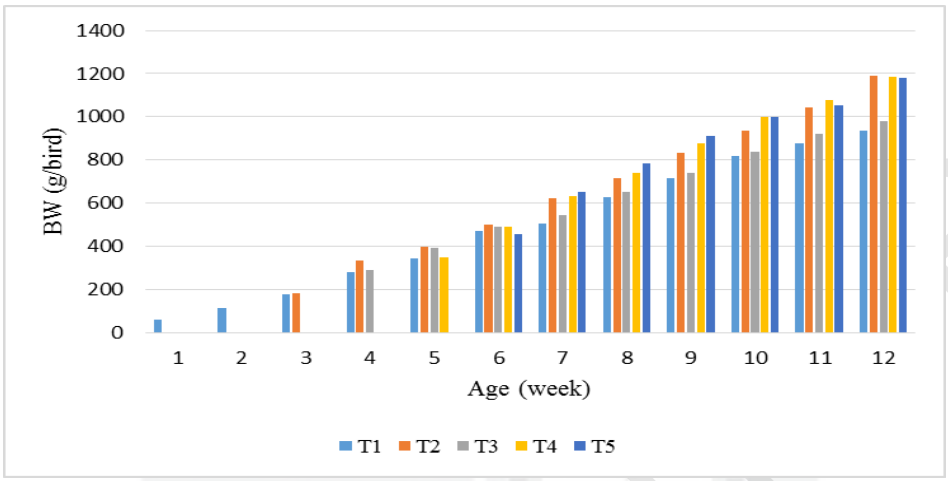


Figure 4.5: Graph of females' body weight effectiveness according to the proceeding time of uropygialectomy.

Appendix 4.6

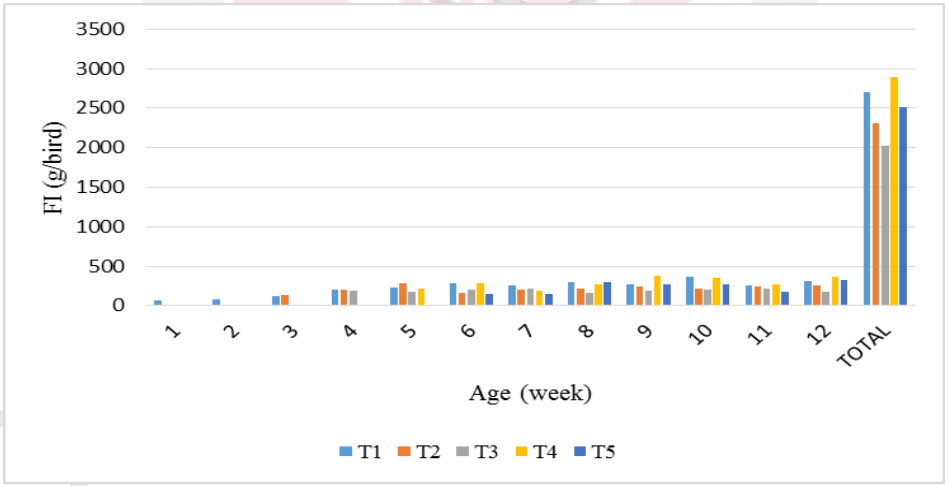


Figure 4.6: Graph of males' feed intake effectiveness according to the proceeding time of uropygialectomy.

Appendix 4.7

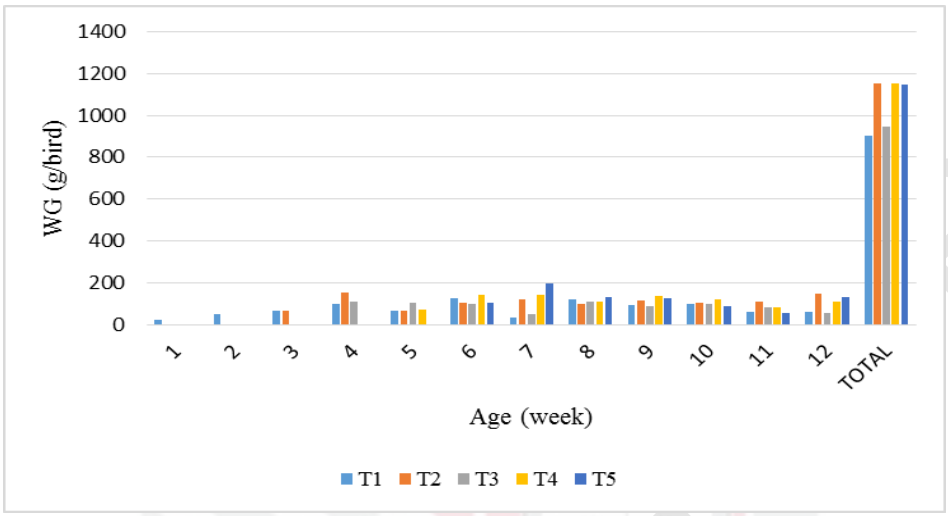


Figure 4.7: Graph of females' weight gain effectiveness according to the proceeding time of uropygialectomy.

Appendix 4.8

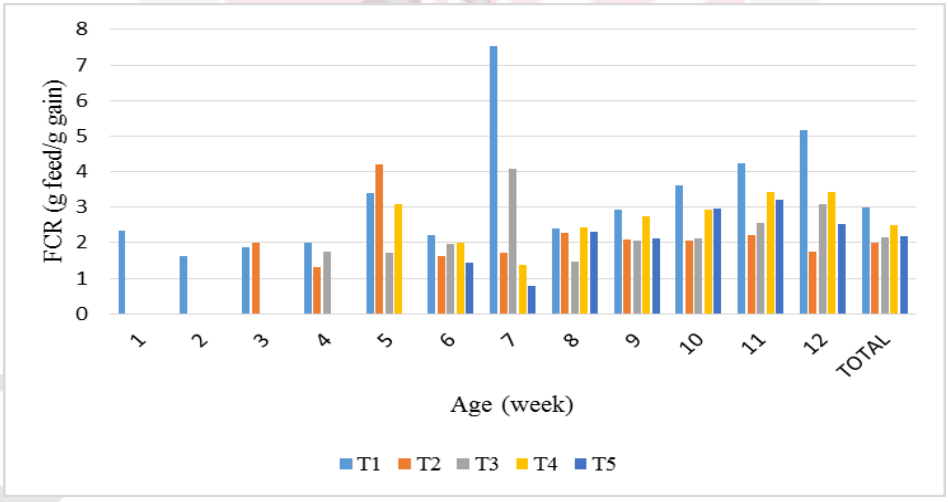
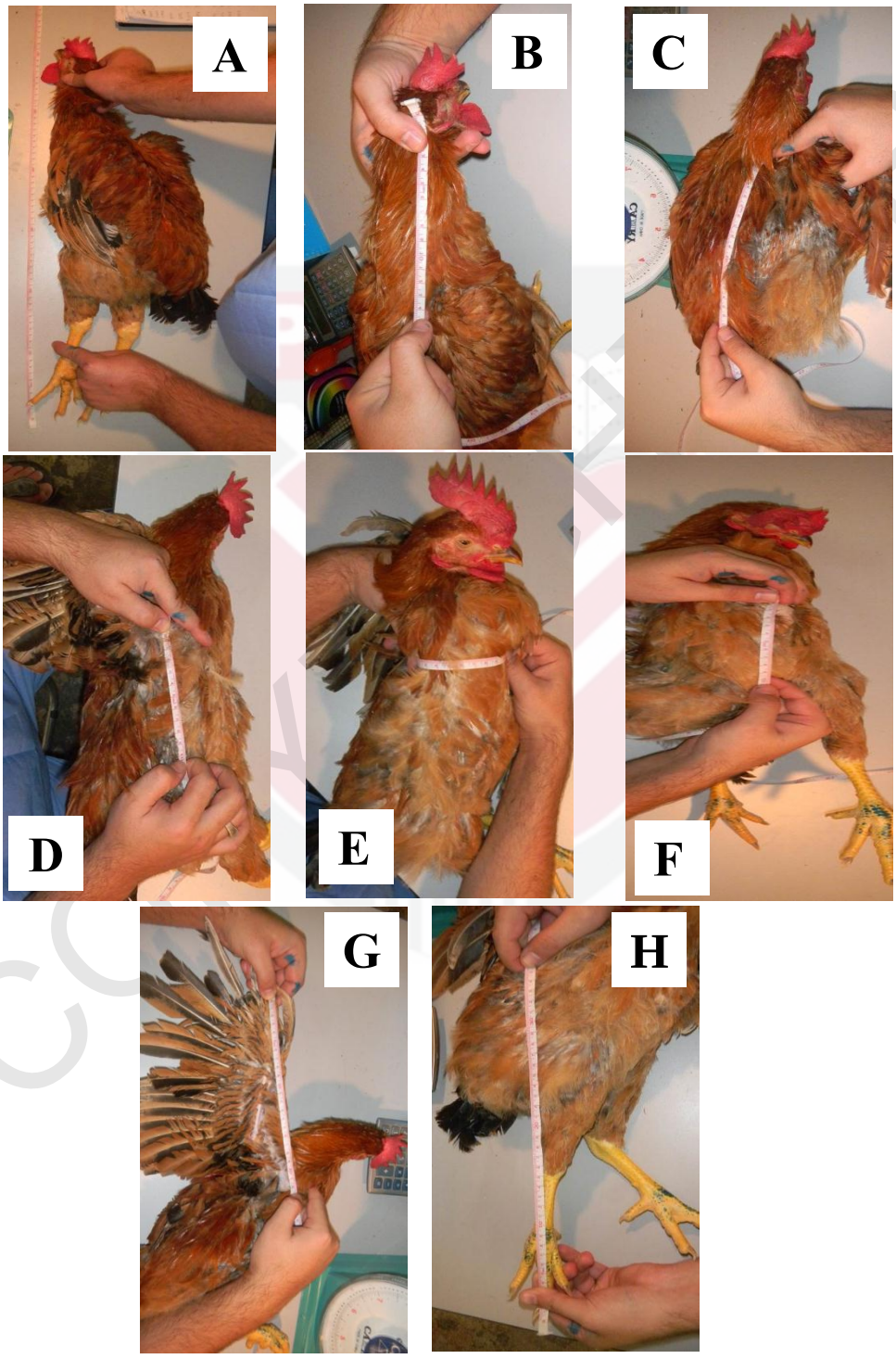


Figure 4.8: Graph of females' feed conversion ratio effectiveness according to the proceeding time of uropygialectomy.

CHAPTER 6

Appendix 6.1



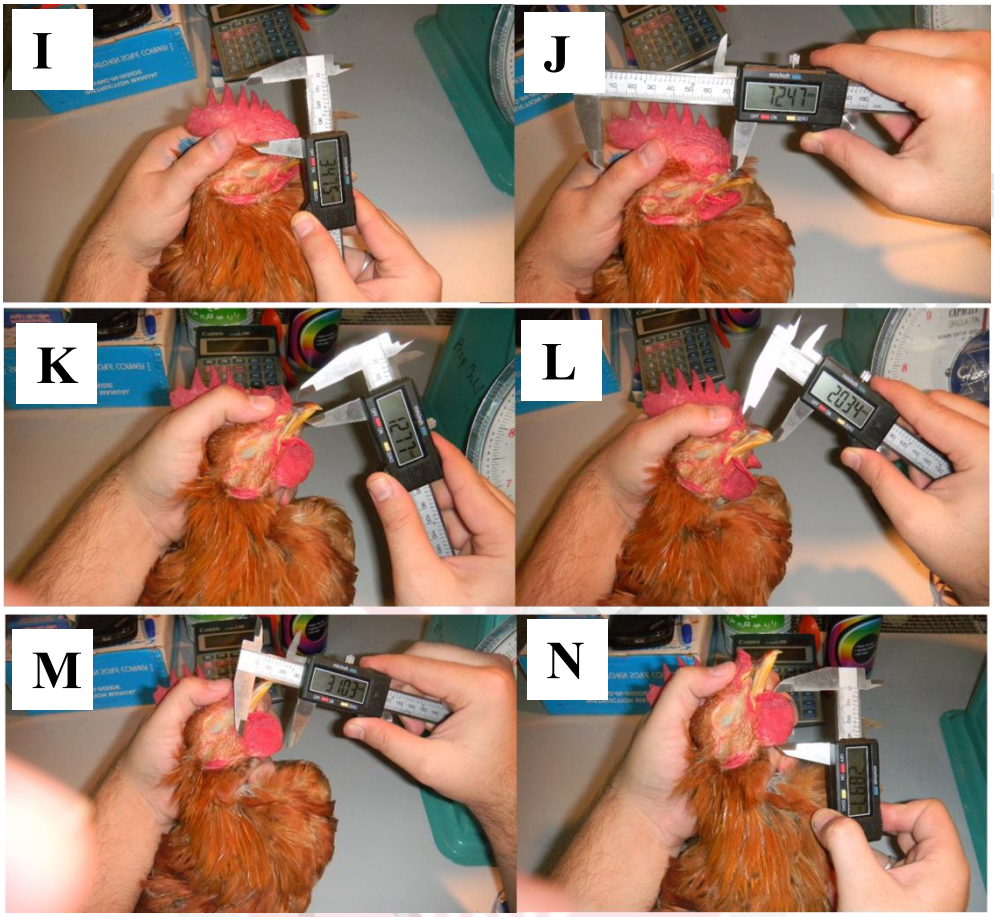
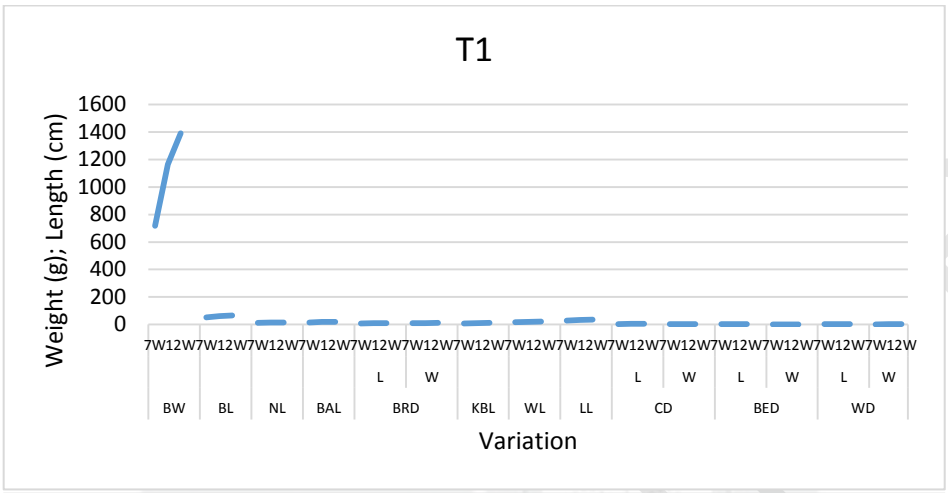


Figure 6.1: Photographs of external morphological characteristics measuring process. Bird length (BL): 2.1A; Neck length (NL): 2.1B; Back length (BAL): 2.1C; Breast length (BRL): 2.1D; Breast width (BRW): 2.1E; Keel of sternum length (KBL): 2.1F; Fore limbs length (WL): 2.1G; Hand limbs length (legs) (LL): 2.1H; Comb width (CW): 2.1I; Comb length (CL): 2.1J; Beak width (BEW): 2.1K). Beak length (BEL): Length from the tip of the beak until insertion of the beak into the skull (Appendix 2.1L; Wattles' width (WAW): 2.1M; Wattles' length (WAL): 2.1N.

Appendix 6.2


T1
Weight (g); Length (cm)
1600
1400
1200
1000
800
600
400
200
0
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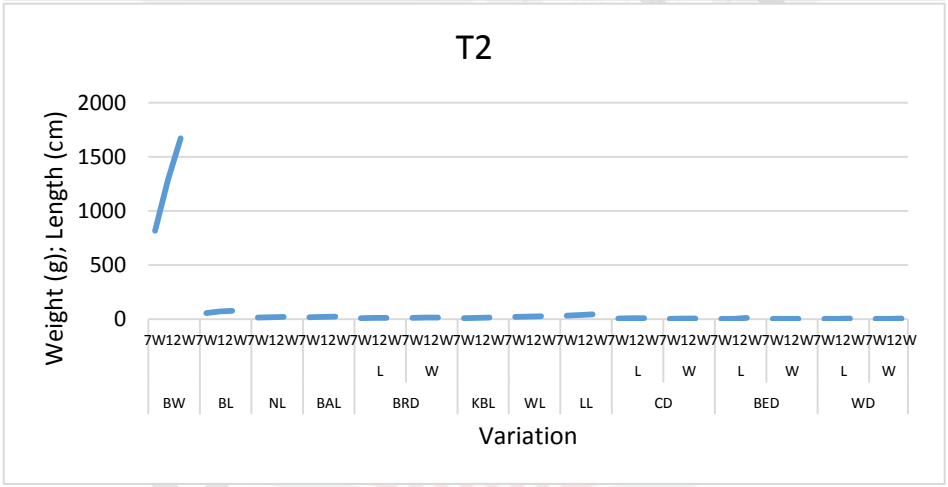
L W L W L W L W
BW BL NL BAL BRD KBL WL LL CD BED WD
Variation


T2
Weight (g); Length (cm)
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1500
1000
500
0
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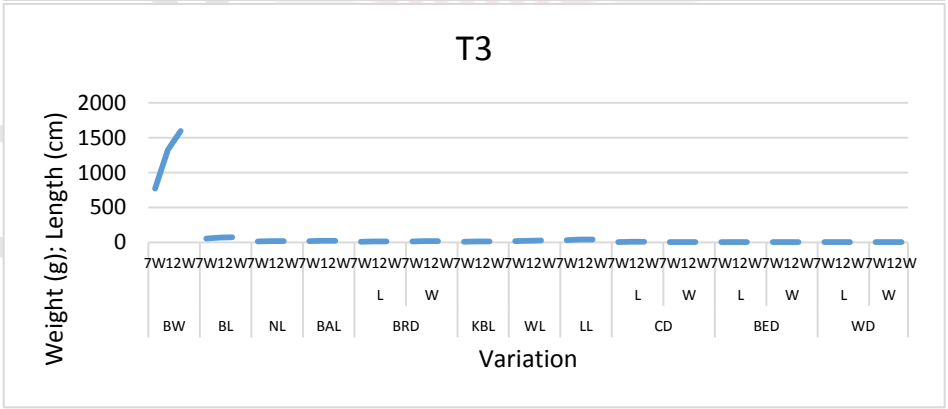
L W L W L W L W
BW BL NL BAL BRD KBL WL LL CD BED WD
Variation


T3
Weight (g); Length (cm)
2000
1500
1000
500
0
7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W7W12W
L W L W L W L W
BW BL NL BAL BRD KBL WL LL CD BED WD
Variation

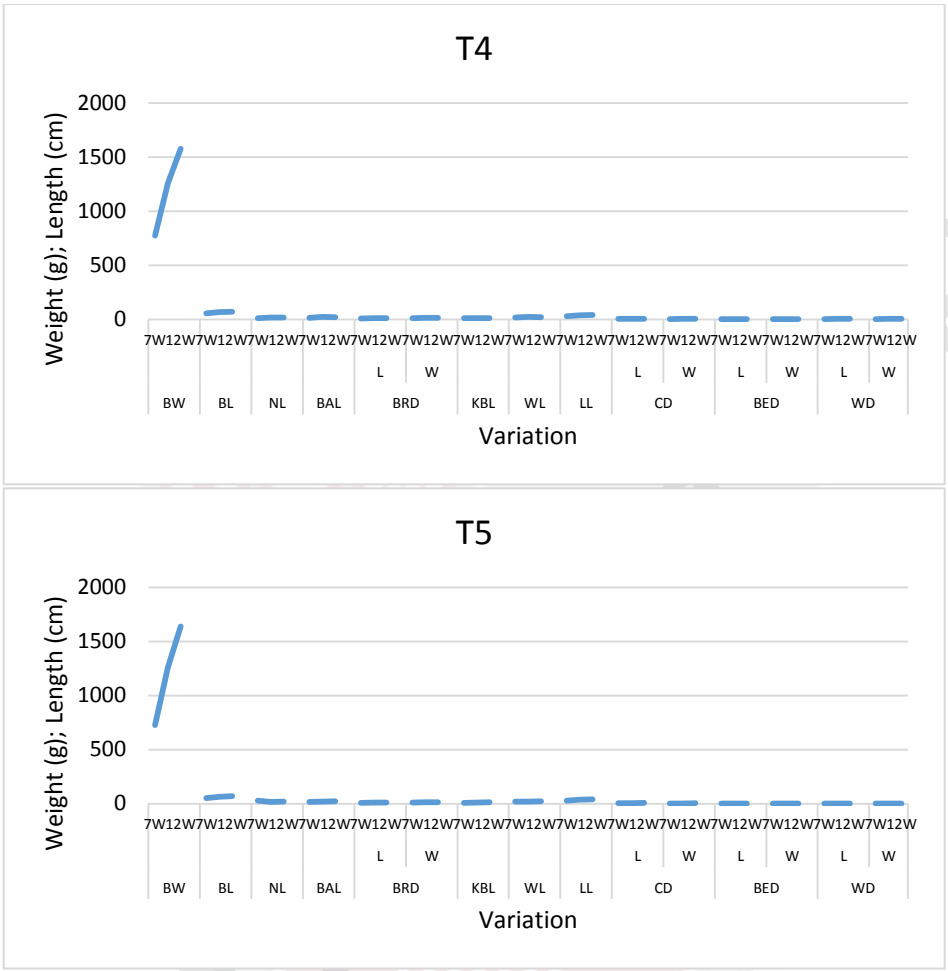


Figure 6.2: Graph of linear development of the morphological characteristics based on the age of measurement (7, 10, 12 weeks). BW: Bird weight; BL: Bird length; NL: Neck length; BAL: Back length; BRD: Breast diameters; KBL: Keel bone length; WL: wing length; LL: Leg length; CD: Comb diameters; BED: Beak diameters; WAD: Wattle diameters; L: Length; W: Wide.

CHAPTER 7

Appendix 7.1

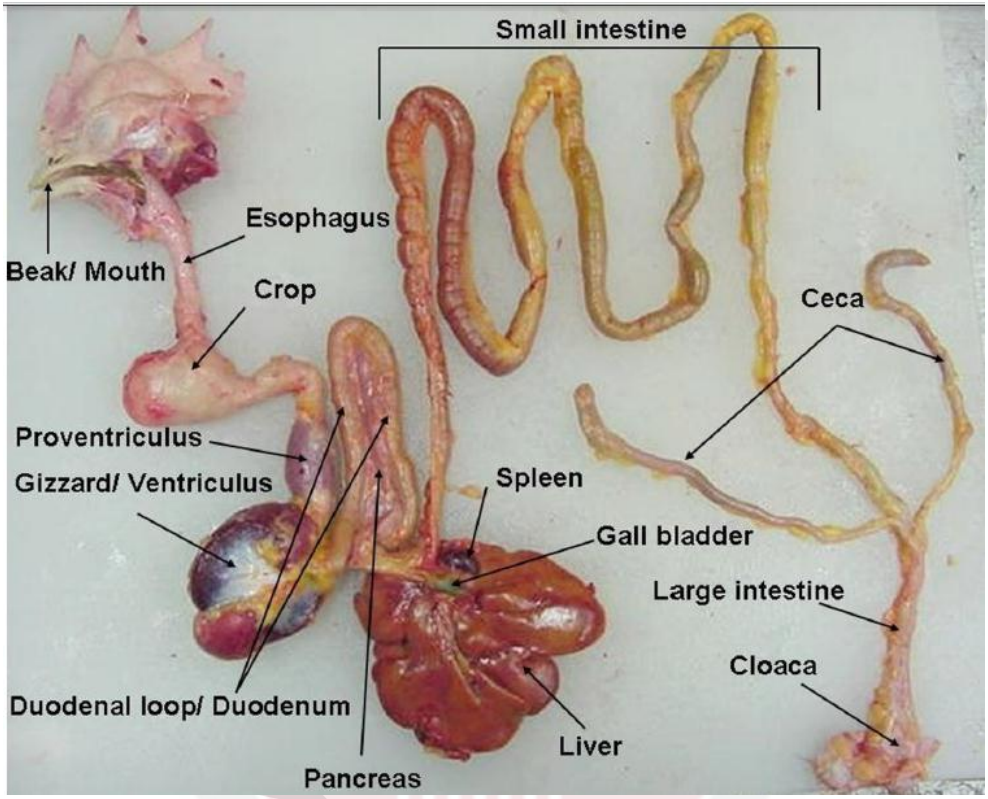


Figure 7.1: Parts of the digestive tract of a chicken (McLelland, 1990).

CHAPTER 8

Appendix 8.1

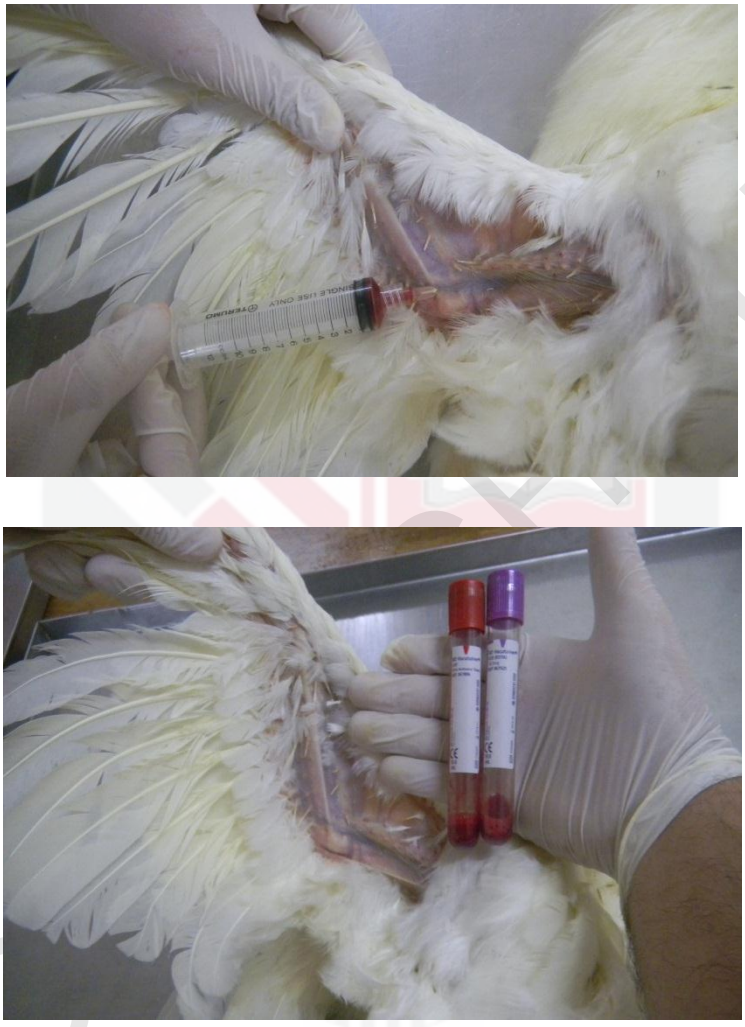


Figure 8.1. Photographs of blood collection method.

Appendix 8.2

CHICKEN GROWTH HORMONE (GH) ELISA Kit

Catalog Number. CSB-E09866Ch
For the quantitative determination of chicken growth hormone (GH) concentrations in serum, plasma, tissue homogenates.

PRINCIPLE OF THE ASSAY
This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with an antibody specific to GH. Standards or samples are added to the appropriate microtiter plate wells with Biotin-conjugated GH. A competitive inhibition reaction is launched between GH (Standards or samples) and Biotin-conjugated GH with the pre-coated antibody specific for GH. The more amount of GH in samples, the less antibody bound by Biotin-conjugated GH. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Substrate solution is added to the wells and the color develops in opposite to the amount of GH in the sample. The color development is stopped and the intensity of the color is measured.

DETECTION RANGE
625 pg/ml-10000 pg/ml.

SENSITIVITY
The minimum detectable dose of chicken GH is typically less than 312.5 pg/ml. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest chicken GH concentration that could be differentiated from zero. It was determined the mean O.D value of 20 replicates of the zero standard added by their three standard deviations.

SPECIFICITY
This assay has high sensitivity and excellent specificity for detection of chicken GH. No significant cross-reactivity or interference between chicken GH and analogues was observed.
Note: Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between chicken GH and all the analogues, therefore, cross reaction may still exist.

PRECISION
Intra-assay Precision (Precision within an assay): CV %< 15% Three samples of known concentration were tested twenty times on one plate to assess.
Inter-assay Precision (Precision between assays): CV %< 15% Three samples of known concentration were tested in twenty assays to assess.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Reagents	Quantity
Assay plate	1(96 wells)
Standard	5 x 1 ml
Conjugate	1 x 6 ml
HRP-avidin	1 x 6 ml
Wash Buffer (20 x concentrate)	1 x 15 ml
Substrate A	1 x 7 ml
Substrate B	1 x 7 ml
Stop Solution	1 x 7 ml
Adhesive Strip (For 96 wells)	4
Instruction manual	1

STANDARD CONCENTRATION

Standard	S1	S2	S3	S4	S5
Concentration (pg/ml)	625	1250	2500	5000	10000

STORAGE

Unopened kit	Store at 2 - 8°C. Do not use the kit beyond the expiration date.
Opened kit	May be stored for up to 1 month at 2 - 8° C.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 600 nm - 630 nm.
- An incubator which can provide stable incubation conditions up to 37°C±0.5°C.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Absorbent paper for blotting the microtiter plate.

- 100 mL and 500 mL graduated cylinders.
- Deionized or distilled water.
- Pipettes and pipette tips.
- Test tubes for dilution.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

- **Serum:** Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 ×g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
- **Plasma:** Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 ×g at 2-8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
- **Tissue Homogenates:** 100mg tissue was rinsed with 1X PBS, homogenized in 1 ml of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g, 2 - 8°C. The supernate was removed and assayed immediately. Alternatively, aliquot and store samples at -20°C or -80°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.

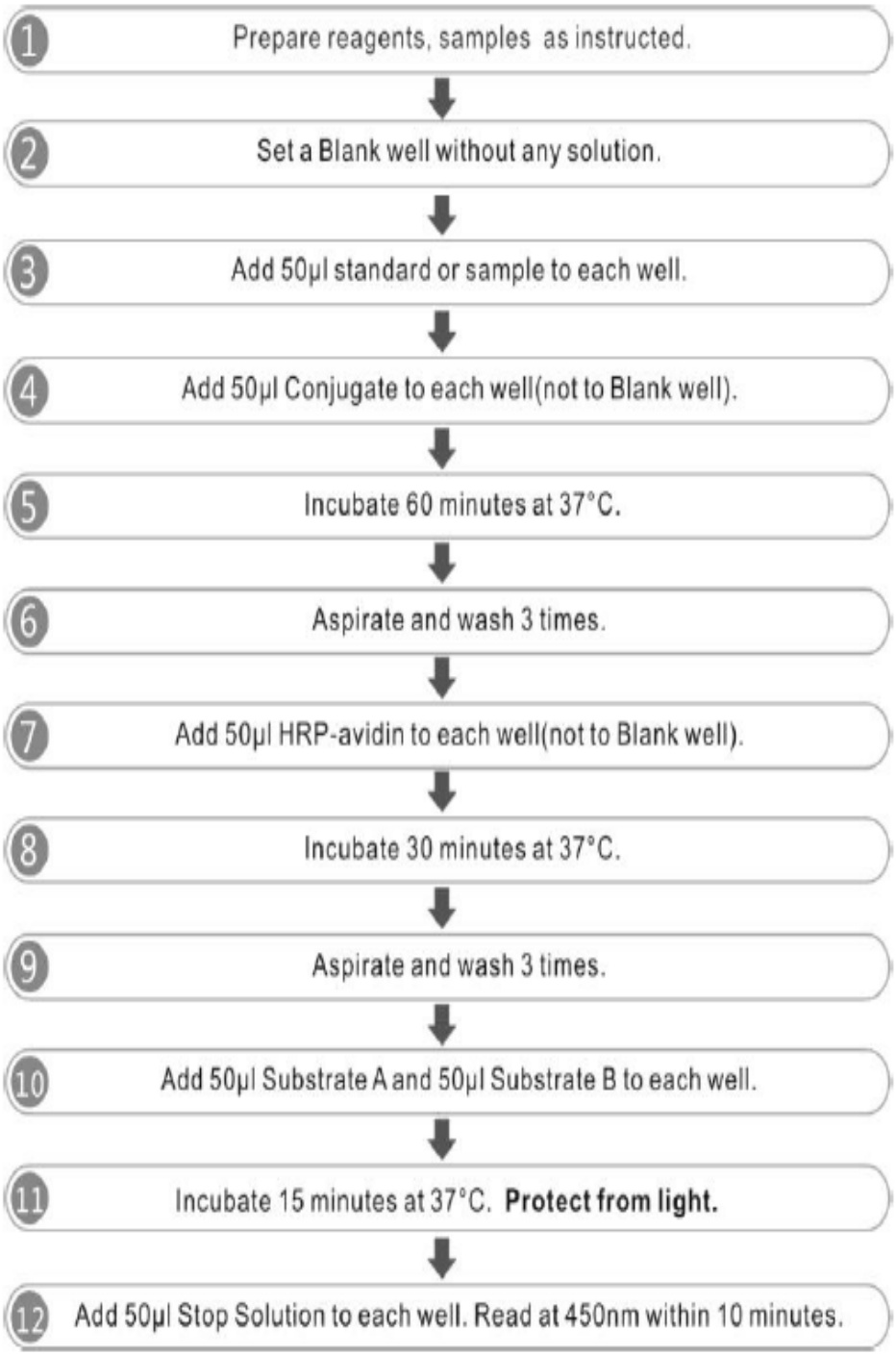
1. Prepare all reagents and samples as directed in the previous sections.
2. Determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. Set a Blank well without any solution.
4. Add 50µl of Standard or Sample per well. Standard need test in duplicate.
5. Add 50µl of Conjugate to each well (not to Blank well). Mix well and then incubate for 60 minutes at 37°C.
6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 10 seconds, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating ordecanting. Invert the plate and blot it against clean paper towels.
7. Add 50µl of HRP-avidin to each well (not to Blank well). Mix well and then incubate for 30 minutes at 37°C.
8. Repeat the aspiration/wash process for three times as in step 6.

9. Add 50µl of Substrate A and 50µl of Substrate B to each well, mix well. Incubate for 15 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark.
10. Add 50µl of Stop Solution to each well, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 10 minutes, using a microplate reader set to 450 nm.

Note:

1. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments.
2. Samples or reagents addition: Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall as possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.
4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading. When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
5. Controlling of reaction time: Observe the change of color after adding Substrates (e.g. observation once every 10 minutes). Substrates should change from colorless or light blue to gradations of blue. If the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. Substrates are easily contaminated. Substrates should remain colorless or light blue until added to the plate. Please protect it from light.
7. Stop Solution should be added to the plate in the same order as the Substrates. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrates.

ASSAY PROCEDURE SUMMARY



CALCULATION OF RESULTS

Using the professional soft "Curve Expert 1.3" to make a standard curve is recommended, which can be downloaded from our web.

Average the duplicate readings for each standard and sample and subtract the average optical density of Blank. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the GH concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Appendix 8.3

Chemical preparation for light microscopy

1- 10% Neutral Buffered Formalin (NBF)

Formalin (37-40% Formaldehyde)	100.00 ml
Sodium Phosphate Monobasic	4.00 g
Sodium Phosphate Dibasic (anhydrous)	6.50 g
Distilled water	900.00 ml

Dissolved the sodium phosphate monobasic and sodium phosphate dibasic in 900 ml distilled water and add 100 ml of 40% formalin.

2- Harris Hematoxylin and Eosin

Hematoxylin	1.0 g
Potassium alum (KAI (SO4)2.12H2O)	20 g
Mercuric oxide	0.5 g
Ethyl alcohol	10 ml
Distilled water	200 ml

Dissolve hematoxylin in ethyl alcohol. Dissolve potassium alum in water and boil. Add hematoxylin and boil ½ minute. Add mercuric oxide. Cool rapidly. Add a few drops of acetic acid.

Eosin

Eosin Y (C.I. 45380)	1.0 g
Potassium dichromate	0.5 g
Saturated aqueous picric acid	10.0 ml
Absolute ethyl alcohol	10.0 ml
Distilled water	80.0 ml
Acetic acid	1 drop

Procedure

1- Xylene: 2-3 minutes (two changes).
2-absolute alcohol: 2-3 minutes.
3- 95% alcohol: 2-3 minutes.
4- 80% alcohol: 2-3 minutes.
5- 75% alcohol: 2-3 minutes.
6- Running water: 3 minutes.
7- Harris hematoxylin: 2-5 minutes. Check after 1 minute.
8- Running water: 3-5 minutes.
9- Counterstain: eosin or 0.5% aqueous phloxin B.
10-Dehydration through series of alcohol 70%, 95%, absolute alcohol.
11- Xylene: 2-3 minutes two changes.
12- Monting medium and add cover glass.

BIODATA OF STUDENT

The student was born on March 3, 1983 in Baghdad, Iraq. He attended his primary and secondary school in his hometown, Baghdad. He pursued his study in Veterinary Medicine at University of Baghdad. He completed his DVM degree in 2006, ranking 85th out of 177. At the same year, he started his post-graduate study for MSc in the Department of Anatomy and Histology at the Faculty of Veterinary Medicine, University of Baghdad, and obtained the degree in 2008. After that, he was directly appointed in Department of Animal Production of the Agriculture faculty. Thereafter, he served at Agriculture Faculty as an assistant lecturer until 2012, and then he was promoted to lecturer. His interest in academic and research fields led him to continue his study for a PhD degree in Anatomy and Histology at University Putra Malaysia (UPM) under the supervision of Dr. Lokman Hakim Idris at Faculty of Veterinary Medicine, University Putra Malaysia in 2013.

LIST OF PUBLICATIONS

Jawad, H.S., Idris, L.H.B., Naji, S.A., Bakar, M.B., and Kasim, A.B. (2015). Partial Ablation of Uropygial Gland Effect on Production Performance of Akar Putra Chicken. *International Journal of Poultry Science*, 14(4), 213-221.

Jawad, H.S., Idris, L.H.B., Bakar, M.B., and Kasim, A.B. (2015). Anatomical Changes of Akar Putra Chicken Digestive System after Partial Ablation of Uropygial Gland. *American Journal of Animal and Veterinary Sciences*, 10(4), 217-229.

Jawad, H.S., Idris, L.H.B., Naji, S.A., Bakar, M.B., and Kasim, A.B. (2015). Anatomical Changes of Akar Putra Chicken Digestive System after Partial Ablation of Uropygial Gland. Paper participated as poster in the *2^{ed} World Veterinary Poultry Association and World Poultry Science Association (Malaysia Branch) Scientific Conference*, "Enhancing Innovation in Poultry Health and Production", ISBN 978-983-2408-28-4 21-22th September 2015. Kuala Lumpur convention Centre.

Jawad, H.S., Lokman, I.H., Zuki, A.B.Z., and Kasim, A.B. (2016). Partial ablation of uropygial gland effects on growth hormone concentration and digestive system histometrical aspect of Akar Putra chicken. *Poultry Science*, 95(4): 966-973. doi: 10.3382/ps/pev444.

Jawad, H.S., Lokman, I.H., Zuki, A.B.Z., and Kasim, A.B. (2016). Partial ablation of uropygial gland effects on carcass characteristics of Akar Putra chicken. *Poultry Science*. doi: 10/3382/ps/pev125.

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