IN-OVO INJECTION OF TONGKAT ALI (*Eurycoma longifolia*) EXTRACT ON THE PRE-HATCH DEVELOPMENT OF CHICKEN EMBRYO

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ABSTRACT

This study was conducted to investigate the effects of in-ovo administration of Tongkat Ali (TA) on the mortality and pre-hatch development of chicken embryo. The fertile eggs of cross-bred village chicken were injected with liquid TA that diluted from powder form at the beginning of incubation. Initially, a dosage response study of TA (0 mg, 0.1 mg, 1 mg and 10 mg/ 100g egg) was conducted. Then, the effects of 0.1 mg/ 100g egg TA were investigated on the embryonic mortality and body parts development until 19th day of incubation. Results showed a toxic effect of TA on the embryonic survivability and development, and the LD_{50} was found at around 10 mg/ 100g egg in comparison of control mortality. At 88 to 96 hours of incubation, the 0.1 mg/ 100g egg TA injection (38 somites) significantly reduced the number of somite in the embryo as compared to that in the control (42 somites). At 19th day of incubation, this dosage also attributed an insignificantly lower embryonic weight (53.38 % to egg weight) than in the control (70.50 % to egg weight). The dosage applied showed the insignificant difference on the wing length at 9th, 16th and 19th day of incubation. For limb length parameter, the TA dosage had showed a significant higher in 19th day TA injected chick embryo. Relatively much earlier and huge feather-follicle development was observed in the TA injected embryos compared to control. On the day 19th of incubation, 100 % TA exposed chicks produced a traceable vocal sound along with pipping mark in the shell, but these phenomena were completely absent in the chick of control group. This study suggested that administration of TA through in-ovo injection route could bring both the positive and negative effects in developing chicken embryo, however, more studies are required to elucidate the mechanisms of TA effect including the embryonic sex-specificity in different poultry species.

PENDEDAHAN TONGKAT ALI (Eurycoma longifolia) EXTRACT SECARA "IN-OVO" DALAM PEMBANGUNAN PRA PENETASAN EMBRIO AYAM

ABSTRAK

Kajian ini telah dijalankan untuk menyiasat kesan pentadbiran "in-ovo" Tongkat Ali (TA) pada pembangunan dan kematian pra-penetasan embrio ayam. Telur-telur yang subur dari kacukan ayam kampung telah disuntik dengan cecair TA dari bentuk serbuk pada awal inkubasi. Pada mulanya, kajian tindak balas dos TA (daripada 0.0 mg kepada 10 mg/ 100g telur) telah dijalankan. Kemudian, kesan daripada 0.1 mg/ 100g telur TA telah dikaji dalam aspek kematian embrio dan pembangunan bahagian badan sehingga 19 hari inkubasi. Keputusan menunjukkan kesan toksik TA pada kemandirian embrio dan pembangunan, dan kira-kira 10 mg/ 100g telur telah diperhatikan sebagai LD_{50}. Dalam 88- 96 jam inkubasi, 0.1 mg/ 100g telur TA suntikan didapati mengurangkan bilangan somite (38 somites) secara ketara dalam embrio berbanding yang dalam kawalan (42 somites). Pada hari ke-19 inkubasi, dos ini juga menunjukan berat badan embrio yang lebih rendah (53.38 % kepada berat badan telur) dalam perbandingan dengan kawalan (70.50 % kepada berat badan telur). Dos TA yang digunakan telah menunjukkan perbezaan secara tidak ketara pada kepanjangan sayap dalam hari ke-9, -16 dan -19 masa inkubasi. Untuk parameter kepanjangan kaki, dos TA telah menunjukkan ketinggian yang ketara pada hari ke-19 masa inkubasi.Pertumbuhan bulu-folikel yang lebih awal dan cepat telah diperhatikan dalam TA pendedahan embrio berbanding dalam kawalan. Pada ke-19 hari inkubasi, 100 % TA pendedahan anak ayam menghasilkan bunyi vokal boleh dikesan bersama-sama dengan pipping tanda di shell, tetapi fenomena ini adalah sama sekali tidak hadir dalam anak ayam dari kumpulan kawalan. Kajian ini mencadangkan bahawa pentadbiran TA melalui "in-ovo" laluan suntikan boleh membawa kedua-dua kesan positif dan negatif dalam membangunkan embrio ayam, walaubagaimanapun, lebih banyak kajian diperlukan untuk menjelaskan mekanisme kesan TA termasuk embrio seks-ketentuan dalam spesies haiwan ternakan yang berbeza.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, UNITS AND ABBREVIATIONS

ANOVA	Analysis of variance
CAM	Cell-adhesion molecule
cGMP	Cyclic guanosine phosphate
cm	Centimetres
ECM	Extracellular matrix
EL	*Eurycoma longfolia* Jack
FCR	Feed conversion rate
FPL	Faculty of Sustainable Agriculture
g	Grams
HG 36	Livestock Production Programme
kg	Kilograms
LD_{50}	Lethal dosage
m	Metres
m^2	Square Metres
mg	Micrograms
ml	Millilitres
mm	Millimetres
N	Number of population
NaCl	Sodium chloride
NaK ATPase	Sodium-potassium adenosine triphosphatase
P	Probability
PEPT1	Oligopeptide transporter
SGLT-1	Sodium dependent glucose transporter 1
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Science
TA	Tongkat Ali
UMS	Universiti Malaysia Sabah
Zn	Zinc
µg	Micrograms
°c	Degree Celsius
%	Percentage

LIST OF FOMULAE

Formula		Page
3.1	Percentage of Total Embryo Mortality(a) $a\,(\%) = \frac{\text{Total Death Embryos}}{\text{Total Fertile Eggs}} \times 100\%$	19

CHAPTER 1

INTRODUCTION

1.1 Background

Eurycoma longifolia Jack (EL) or commercially known as Tongkat Ali (TA) in Malaysia, Pasak Bumi in Indonesian, Piak and Tung Saw Thailand, and Cay ba binh in Vietnam is a famous medicinal plant in the family of Simaroubaceae (Effendy *et al.*, 2012). TA has been widely distributed in South East Asian countries such as Malaysia, Vietnam, Indonesia and Thailand and, it reserves a great range of medical property as general health tonic including the improvement in physical and mental energy levels as well as the overall quality of life. TA is also well-known to various ethnic groups of Malaysian for its male sexual health enhancer effects.

Nowadays, TA can be found in varieties of dietary supplements with the intention to improve libido and energy as well as restore testosterone level which is indirectly act as growth rate indicator. Several studies reported that TA contains a small group of peptides, referred to as "eurypeptides" that stimulates the release of testosterone from its binding protein, sex-hormone-binding-globulin. Through this way, TA can be considered as the "maintainer" and "restorer" of testosterone level in one's body. In the experimental condition, TA feeding on numerous rodents has restored the testosterone level (Talbott *et al.*, 2013).

The study of developmental biology or pre-hatch development of embryology in chicken is one of the major parts, not only in poultry industry but also on specific effects of the product of biomedical research as nutritional supplement, which largely coincide human foetus development (Gelder and VanBelanger, 1989). However, nowadays, there is still a limitation in the information toward hatching for development, physiology and metabolism of poultry embryos. An improvement in the gain of body

weight during last two decades has successfully made through the pre-hatch development for poultry production (Hulet, 2007). It was suggested that any elements that supports or limits the growth during the incubation period reserved a marked effect on overall performance of the flocks. Therefore, poultry researchers are emphasized on advancements made during incubation period and embryogenesis for future gains in related industry.

Village chicken marked the growing economic importance in sustaining the demands of local community and providing the alternative healthier animal protein source as the requirement of high quality nutrients (proteins and micronutrients) as one of the dietary supplements in providing a healthy lifestyle is increasing tremendously. The parameters of body weight gain, growth rate and mortality percentage should be taken into consideration in production, supplying and marketing of village chickens to local and international communities. As recorded by Awan (1993), on the basis of data collected from the Department of Veterinary Services Malaysia, the estimated population 6,500,000 of village chickens to be recorded in Malaysia at the year of 1993. In the comparison with domestic consumption of chicken meat, it was increasing trend from 577,900 tonnes in 2001 to more than 918,000 tonnes in 2011. Consequently, village chicken production has great potential in satisfying at least part of the demands through increased productivity and reduced wastage and losses through low-input production systems (Soniaya and Swan, 2004).

"Most of the people think that the first meal that the chick consumes is when it hatches, but in fact the first meal is that when embryo consumes the amniotic fluid as it hatches" (Ferket and Uni, 2011). In-ovo feeding is able to affect the performance of hatching chicks by providing the feed to the developing embryo through the amniotic fluid. In another word, in-ovo feeding can be defined as the injection of nutrients to the amnion during the incubation process of egg. As mentioned in several scientific studies, in-ovo feeding reserved some significant advantages which included the improvement in digestion capacity and immune to foreign enteric agent, increasing the growth rate and feed efficiency, reducing the post-hatch mortality and morbidity as well as improve in muscle development. As mentioned by Pal *et al.*, slightly amendment in nutritional physiology (in-ovo technology of feeding) during the embryonic development is significant as any changes during this period can have effect

on metabolism with consequently impact of body composition and development by changes in nutrient metabolism and utilization. Therefore, in-ovo feeding technology has established an advance era in the science of perinatal nutrition which provides a wider opportunity for higher production efficiency in term of body weight gain and improvement of feed efficiency and animal welfare.

1.2 Justification of the Study

This experiment will be conducted with in-ovo injection of Tongkat Ali (TA), *Eurycoma longifolia*) extract to fertile eggs with an aim to increase the body weight and reducing of the sexual dimorphism in village chicken. As one of the Bachelor Degree students of Livestock Production (HG36), the conduction of this experiment using village chicken, which is one of the major livestock species in Malaysia, is justifiable under the Faculty course and syllabus circumference.

Nowadays, the chicken has high potential in ensuring the production for alternative animal protein throughout the year. According to the estimation of the United States Department of Agriculture, the world chicken meat production (including table birds and culled layers) is likely to exceed 93 million tonnes in 2013. In Africa, village poultry contributes over 70% of poultry products and 20% of animal protein intake (Kitalyi, 1997). Therefore, the body weight gain, feed conversion ratio and mortality rate in chicken are those vital economic parameters in supplying the eggs and fresh meat products in the market. Under this research, there is a desire in developing an alternative way of producing the heavier chicken with higher feed conversion efficiency and low mortality for increasing population and requirement of market. Therefore, this experiment is highly justifiable especially in term of production for meeting up the local and international demands.

TA has been widely distributed in some South East Asian countries such as Malaysia, Thailand and Indonesia. As a local product, this medicinal plant is easily available in Malaysian local market for manufacturing the dietary supplement of chicken industry. The medicinal effect of TA for increasing the testosterone level in human and livestock animals had been previously studied and well proven in some scientific research (Zanoli *et al.*, 2009). However, the effect of in-ovo route administration of TA on village poultry is still underdetermined.

Food security has been appeared as a critical global issue recently. This experiment is justifiable to address the stated issue locally and globally. Since the animal based foods are rich in energy, protein and micronutrients which give the higher bioavailability than plant source, this is possible that the food security can be achieved more efficiently when people have optimal access of food from village chicken production. Besides, the protein based food risk can be reduced with the application of this concept in the field animal food production.

1.3 Objective

This study was conducted to investigate the effects of in-ovo Tongkat Ali (TA) exposure in the cross-bred village chicken on:

1. the embryonic mortality and weight gain, and
2. development of different body parts at pre-hatch period.

1.4 Hypothesis

H_0 : In-ovo injection of Tongkat Ali (*Eurycoma longifolia*) extract has no significant effect on the embryonic mortality and body parts development in cross-bred village chicken during the pre-hatch development.

H_1 : In-ovo injection of Tongkat Ali (*Eurycoma longifolia*) extract has significant effect on the embryonic mortality and at least one of the one of the body parts development in cross-bred village chicken during the pre-hatch period.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Tongkat Ali (*Eurycoma longifolia*)

Tongkat Ali (TA), scientifically known as *Eurycoma longifolia* in the family of Simaroubaceae is one of the most sought after medicinal herbal plant that native to the jungles of Malaysia, Indonesia and Thailand. It is commonly known as Tongkat Ali in Malaysia and Singapore, Piak and Tung Saw in Thailand, Pasak Bumi in Indonesia and Cay ba binh in Vietnam. From the research studies, the medicinal properties of every parts of TA have been known for century (Ang *et al.*, 2003; Kuo *et al.*, 2003).

2.1.1 General Characteristics of Tongkat Ali

From the scientific name of Tongkat Ali (TA), it reviews some characteristics of this medicinal plant. In Greek, "eurys" means broad while "come" means "a tufted bush", therefore the combination words of Eurycoma denotes to the growing habitat of its compound leaves which spirals out at the tip of its slender trunk in a broad dense rosette. From its original word in Latin, "Longus" means long and "folia" means leaves, so this combination of Longifolia carry the meaning of the characteristics of long and slender compound leaflets that often reaching 20 centimetres in length on TA plant (Forest Research Institute Malaysia, 2014).

TA is a type of shrub herb that can grows up to 15 meters in height and it takes about 20 years to reach their maturity state in general. TA leaves (20 to 40 cm) are green in colour, spirally arranged and pinnate in shape with about 13 to 41 leaflets. The flowers colour of this tree are varying from pink to reddish pink and it is dioecious in which male and female flowers are blooming on different trees. TA plant bears the originally green obloid shaped fruits with the length up to 2 cm and 1 cm in width and

simultaneously turning from red to dark red when they are ripen. TA is more preferable to grow on the acid and sandy soils at the latitude up to 700 m above sea level. This medical plant can be propagated through the seeds that sprout over the cultivated ground after one month. The cultivation period for TA is four years and it is susceptible to the caterpillar, *Atteva scrodoxa* and thus control measures must be taken to ensure the production especially during the peak season of production.

2.1.2 Benefits and Uses of Tongkat Ali

In general, Tongkat Ali (TA) is widely used as health tonic that acts as the folkloric medicine for the medical conditions such as malaria, boils, wounds, ulcers, anti-allergy, high blood pressure, tuberculosis, fever, diarrhoea, jaundice and dysentery. TA is also reserved the benefits in increasing energy and metabolism, reduces fatigue, burns fat and increases overall physical condition. Despite of these therapeutic usages, TA is greatly regarded for its purported aphrodisiac properties (Ang *et al.*, 2003). TA preparations are now widely available in the food products market due to the increasing demands for its remarkable health benefits. TA can be processed into additive that brewed with coffee, canned drink as well as animal feed for boosting up the testosterone level in one's body. It can be obtained in the form of raw crude powder without any additional chemical processing by which the root is dried and grinded. The capsule form of TA is also available either with raw crude powder or standardized TA from the extracted active ingredients of defined concentration level.

2.1.3 Pharmacological and Medicinal Values of Tongkat Ali

From the root of Tongkat Ali (TA), a wide range of chemicals such as eurycomanone, eurycomanol, eurycomalactone, canthine-6-one alkaloid, 9-hydroxycanthin-6-one, 14,15β-dihydroxyklaineanone, phenolic components, tannins, quanissoids, and triterpenes had been isolated (Effendy *et al.*, 2012). The presence of these stated chemicals enable TA possess the effective medicinal value in sexual enhancement, antimalarial, antibacterial as well as antitumor. Besides, TA has been well documented to exert antioxidative properties due to its high concentrations of superoxide dismutase (SOD) and it is famously known for its aphrodisiac effect, which is due to its ability to stimulate the production or action of androgen hormones, especially testosterone (Effendy *et al.*, 2012). Several studies showed that TA reserved the ability in increasing

the plasma testosterone level which in turn will increase sperm counts. Besides, it is scientifically found that TA has the capacity and ability to "reverse the inhibitory effects of estrogen on testosterone production and spermatogenesis". At the same time, TA also contained a small group of peptides that referred as "eurypeptides" that stimulating the release of testosterone from its binding protein, sex-hormone-binding-globulin. Through this way, TA can be considered as "maintainer" and "restorer" of testosterone level in one's body (Talbott *et al.*, 2013).

2.1.4 Side Effects of Tongkat Ali

Although TA reserves a wide range of medicinal value, however, the larger dose and incorrect proportion in application of TA will brings some unfavourable side effects. In human, the insomnia state is the most obviously observed. The application of TA as animal feed especially in animal species can bring some behaviour changes when there is the anti-estrogenic action occurred. The high testosterone level and low estrogen level in animal body can cause restless, stress, aggressive and some minor changes in their physiological development (Frank, 2013). Besides, the uses of higher doses of TA in feed can cause the convulsions, shallow breathing and depression as well as sudden death among the animal (Cloe, 2013).

2.1.5 Application of Tongkat Ali in Animal Species

The scientific study that carried out by Awang Ahmad Bin Mohd Yunus, a scientist and lecturer of the Department of International Tropical Forestry, Universiti Malaysia Sabah claimed that the TA has been successfully applied as chicken feed in order for producing a higher protein and low fat content meat. He also said that TA fed chicken (A-yamli) appear to be more healthy, reserved with better taste and do not require extra hormone injection for growing. Furthermore, in some scientific study on the feeding of TA in some animal species, it is observed that there is a significant increase in the body weight gain (Ang and Cheang, 2001). The application of TA as a feed supplement can be justified as it can ensure the production efficiency of broiler chicken in the aspect of increasing in their body weight. However, the feeding of TA still a new trend in animal feeding industry and thus more studies and application should be encouraged in this area.

2.2 Village Chicken as a Target Animal for Feeding Tongkat Ali

The term kampong or village chicken is usually used in Malaysia for describe a mixed breed of various breeds of chickens indigenous to this part of the world. Village chicken can be made up from several breeds which include the jungle fowl (*Gallus gallus*), the Malay, the Sumatran and the Siamese fighting cock. Kasim and Afdal (2007) claimed that the Malaysian indigenous chicken are decedents of the imported modern chicken strains which are well known for their superior body weight and laying capability. The red jungle fowl is classified as the ancestor of all domestic fowls, which reserved a slow growth rate characteristics and its species ranges from northeast India eastwards across Southern China and down into Malaysia and Indonesia (Condon, 2006).

Village chicken or the indigenous chickens are usually owned by individual household and reared in a local backyard system, or also known as scavenging system. The small-scale or commercial village chicken production reserved several advantages which included local genetic adaptation, lower cost of feed input, higher quality of meat produced, easier in disease control plan since adult chicken is more resistant pathogenic effect as well as little veterinary input is required (Ahlers *et al.*, 2009). Nevertheless, certain aspects (farm biosecurity, nutrients management and fencing system) are needed to be considered in prevention of any disease event in the flock.

2.2.1 Physiological and Morphological Development of Village Chicken

The physiological and morphological development of chicken is started at the incubation time of the fertile eggs until they reached their mature age. The observation for the development of chicken can be generally grouped into two phases which are the pre- and post-hatch stages. In the pre-hatch stage, the physiological and morphological development is being determined with the developing embryos inside the incubated eggs, whereas the post-hatch observation will be concentrated on the hatched chicks after incubation. The developmental stages during incubation period are crucial as it indicate the production quality and quantity of day old chick. Hatchability and chick quality during incubation period can be affected by the characteristics of incubating eggs, the incubation condition as well as the condition prevailing between the hatching and the placement of the birds in the broiler farm. The

nutritional aspect during the pre-hatch development is important to ensure the developing embryo can achieve sufficient nutrients for fully growth and development.

2.2.2 Economic Importance of Village Chicken

The worldwide broiler industry has advanced to the point in which every gram of meat has value and this value can be accounted for evaluating breeds for special operation (Hughes, 2013). The worldwide broiler meat production is closely associated with the growing demand for animal protein. This industry had been increased for many years and it is fuelled by a powerful shift in the consumption pattern. From the Global Poultry Trends 2013, Asian countries consumed 40 % of the world's chicken production which is approximately 42.5 million tonnes in 2013. The total world poultry meat consumption had been expanded from 66.4 million tonnes in 2000 to almost 91 million in 2009, it is estimated of more than 106 million tonnes in 2013.

The village chicken production reserved a high potential as an alternative measure for broiler chicken in fulfilling world meat consumption and animal protein demand. However, aspects such as market situation, product demands, investment cost, operational cost and expected revenue of different types of poultry production are vital in the consideration of marketing analyses and economic importance for substitution of village chicken in major meat consumption.

Table 2.1 Estimated population of rural poultry in certain countries

Country	Number of rural poultry ('000)	Village Poultry (Percentage of National Flock)
Africa	1,500,000	70
China	2,000,000	50
Malaysia	13100	30
Philippines	43,000	72
Thailand	120,000	80
Vietnam	196,000	98

Source: Awan, 1993

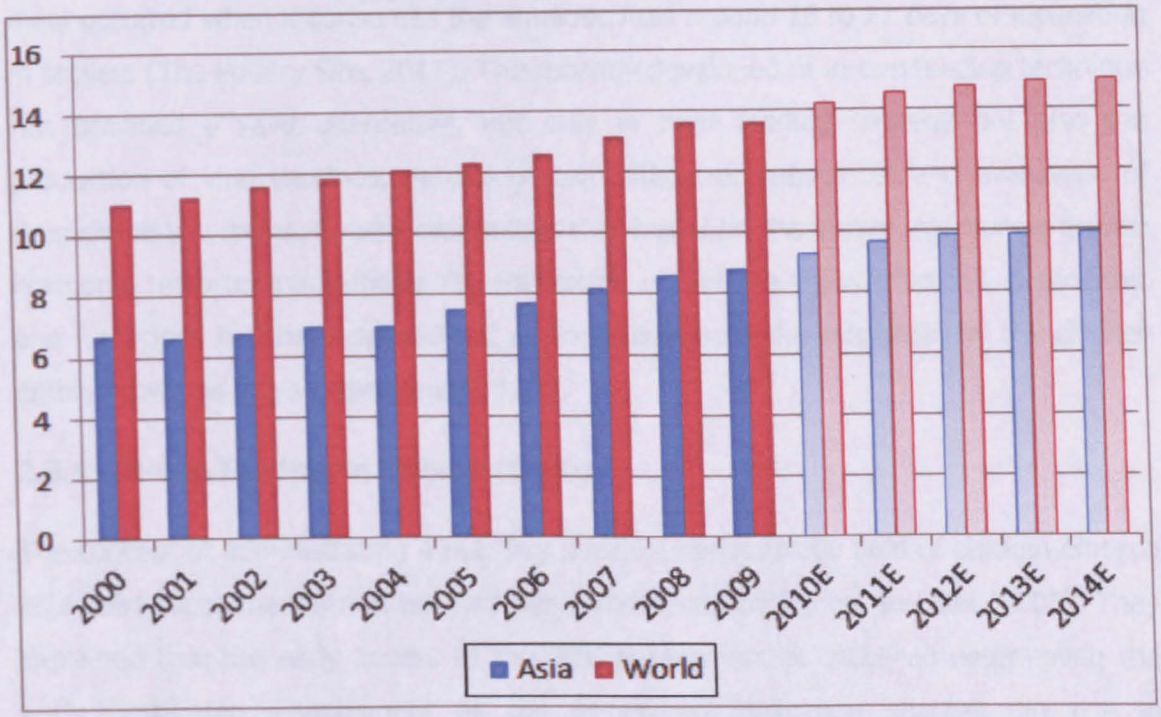

Figure 2.1 The average poultry meat consumption in Asia compared to globes (kg/ person/ year)
Source: The Poultry Site, 2013

Similar to broiler industry, in the economic evaluation for village chicken production, meat yields is appeared as an crucial element that need to take into consideration. The average daily gain, feed conversion ratio (FCR), and mortality percentage are those important parameters in the measuring of meat yields produced by a flock (Hughes, 2013). The increasing meat yields in village chicken flock enable the lower cost in production and allows the sufficient supply of meat products as to fullfill the market demands.

2.3 The Concept of In-ovo Feeding

In-ovo feeding can also be defined as the injection of nutrients to the amnion during the incubation process of egg. The greatest opportunity to facilitate the improvement in hatchability, growth rate and meat production is to change the incubation condition especially the egg internal environment. As claimed by Dr Jean E. de Oliveira in both 2010 and 2007 research studies, chicken embryos are one of the most studied biological organisms as they have short gestation period (incubation) which taken place outside female body. Therefore, the internal fluid of egg must be supplied most of the essential nutrients for chick embryo growth and development. The chick's first

meal occurred when it consumes the amniotic fluid around 18 to 21 days of incubation in broilers (The Poultry Site, 2011). The recently developed of in-ovo feeding technique has provided a valid alternative, not only in their feeding strategy but also the production of viral vaccines, assessing toxic effects of substances and evaluation of drug delivery systems. Several researches that based on the in-ovo injection of growth hormone, testosterone, peptide YY, antibiotics, insulin-like growth factor-I, pathogens, and fungicides had been carried out as to demonstrate the responses of the chicken embryo towards egg's closed environment.

2.3.1 In-ovo Feeding in Village Chicken

The concept of administrating a nutritive solution into amniotic fluid of chicken embryo as a feed supplemental nutrient had been introduced by Ferket and Uni (2003). They explained that the early access to the feed supplement is critical in determining the early post-hatch development as the in-ovo administration enables the rise in hatchability, development of the chicken digestive tract, increasing of body weight and improve in the nutritional status of the hatchling.

One of the new technologies recently introduced to poultry industry is in-ovo feeding and this method is a mechanism for injection of liquid nutrients into the embryos amniotic fluid. This mechanism is beneficial for embryo development because protein and energy are first obtained from the yolk, and derive from the albumen only after 14 days of incubation (Vieira, 2007). "Most of the people think the first meal the chick consumes is when it hatches, but in fact the first meal is when that embryo consumes the amniotic fluid as it hatches." (Ferket and Uni, 2011). As a novel way to feed critical dietary compounds to the embryos, supplying the amnion with appropriate nutrients is essential. The incubation and perinatal period is the critical stage for the nutritional conditioning and it is account for 50 % productive life for 1.2 to 1.4 kg marketable village chicken. The perinatal period is the last four days before the hatching and first four days after hatching in which it is appeared as the most critical stage for the proper growth and development of village chicken.

In-ovo feeding had been carried out by using several types of nutrients in the in-ovo solution such as NaCl, sucrose, maltose, and dextrin beta-hydroxy-beta-methyl butyrate, arginine, egg white protein, and Zn-methionine. Each of the nutritional

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