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Biological Factors Affecting Poultry Embryo Quality

by

Jacob Alhassan Hamidu

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DEDICATION

To my wife Amina, my sons, Nathaniel and Daniel, and my parents for the love they have showed me. I also dedicate the thesis to my primary and junior high school teachers for the educational foundation they gave me.

Abstract

The objectives of this thesis were to investigate some physiological, cellular and molecular changes from genetic selection, flock age and egg storage on embryonic metabolism, cell viability and gene expression in poultry species. Embryonic heat production (EHP), the parameter used to evaluate metabolic status of two turkey genetic strains (Nicholas and Hybrid) and four flock ages (30 wk, 34 wk, 55 wk, 60 wk) were determined. The results showed that embryos of older flocks had higher EHP than all other flock ages. The higher metabolism can cause overheating in embryos from older flocks during the later part of incubation. The EHP values can assist incubator manufacturers and hatchery managers to manage incubation temperatures to increase embryo survival. The EHP was also determined in two Ross 308 broiler breeder egg storage durations (4 d and 14 d) to assess the impact of long-term egg storage on embryonic metabolism. The embryos from 4 d stored eggs had higher body weight and EHP than embryos from 14 d stored eggs. To investigate what could be the fundamental reasons for the metabolic differences, blastoderms from eggs in these egg storage treatments were isolated, dissociated into single cells, stained with fluorescent dyes and subjected to flow cytometry analysis to differentiate between viable (live), apoptotic and necrotic cell populations. Quantitative real time PCR analysis was then used to compare the expression of selected apoptotic genes (*Bak, Bax, Bok, Bcl-2, Bcl-xL*) in blastoderms and embryos from 6 d incubated eggs. While percentage of viable cells decreased significantly, an unregulated amount of apoptosis increased with egg storage duration ($p < 0.0001$). Expression

of pro-apoptotic genes (*Bak*, *Bax*, and *Bok*) were up regulated as storage duration increased at the blastodermal level but all genes were down regulated after 6 d of incubation. This suggests that an increment in egg storage duration can cause activation of apoptotic cell death mechanisms at the blastodermal level, which can be one of the molecular mechanisms that led to the reduced daily embryonic growth and metabolism observed in 14 d stored eggs. The thesis suggests there are molecular and cellular implications of egg storage.

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1. INTRODUCTION

Embryonic development is a dynamic process that requires a fine balance between several factors in order to achieve an optimum embryo quality before proper hatchability results can be realized. These factors include but not limited to genetic selection (Christensen et al., 1999a, 2008; Liptói and Hidas, 2006), inbreeding (Lambert, 1931), parent flock age (Elibol and Brake, 2006; Christensen et al., 2007a, b), nutrition and health of animals (Kenny and Kemp, 2005). Other factors that affect embryonic performance include incubation temperature (Christensen et al., 2004a, b, 2005, 2007c), relative humidity (Christensen et al., 2006a), egg handling procedures and egg storage (Heier and Jarp, 2003; Boerjan and van Riel, 2003; Christensen et al., 2003; Fasenko, 2007). Currently, the attention should also focus on influences due to genetic selection, maternal flock age and or storage of hatching eggs prior to incubation as a result of current industry concerns of decreased embryo viability.

In turkeys the effects of genetic selection and parent flock age on cardiac energy metabolism have been shown to result from differences in embryonic oxygen (O_2) utilization during the later period of incubation (Christensen et al., 2006a). Modern studies have shown that O_2 consumption of embryos could be due to factors such as genetic strain and flock age; however, this was not observed in turkeys (O'Dea et al., 2004; Hamidu et al., 2007). In broiler breeders, it has been shown that differences in embryonic heat production (EHP), the parameter

used as indication of embryonic metabolism, also calculated partly from O_2 consumption were due to differences in genetic strains (Ross 308 versus Cobb 500) and parent flock age. These differences were observed largely during early (1 to 7 d) and late (14 to 21 d) periods of incubation (Hamidu et al., 2007). A careful examination of the two periods showed that they coincided with the two periods of embryonic development where embryonic mortality is normally higher during commercial incubation of broiler and turkey eggs (Tona et al., 2001; Fairchild et al., 2002). Therefore it can be suggested that embryonic metabolism and embryonic mortality may be related. However, research on the effects of gas exchange or embryonic metabolic status at different turkey genetic strains and parent flock ages on embryo development and survival is lacking. Understanding such effect may help to tailor turkey incubation conditions to different strains or flock ages to increase embryo performance and subsequently increase hatchability.

Another important factor that affects poultry productivity economically and biologically before and during incubation is cold storage of fertile or hatching eggs. It is a common practice on breeder farms where egg producers accumulate the eggs to minimize cost of transportation to hatcheries. In addition, hatcheries store eggs to maximize incubator capacity. Although economically and logistically beneficial, cold storage of eggs can increase embryo mortality which can make the initial economic value less attractive (Fasenko, 2007). Importantly, there is more evidence to show that egg storage may have cellular and molecular basis to the reduction of embryonic performance (Foulkes, 1990; Fasenko et al.,

1992; Bakst and Akuffo, 1999; Bakst, 2003). Currently, the following questions continue to be a challenge to many poultry scientists and producers; what happens to blastodermal cells following egg storage and if apoptosis (programmed cell death) and necrosis (injury driven cell death) may be the biological mechanisms responsible for blastodermal cell death during egg storage. And whether prolonged egg storage can trigger negative expression of genes that execute cell death. These mechanisms have the potential to reduce embryo quality. A proper evaluation of cell viability or cell death and a study of some molecular changes or gene expression in developing embryos may explain some of the physiological differences in embryonic development due to cold storage of eggs in broiler and turkey incubation.

In chapter 1 of this thesis, a number of previously accomplished research on genetic selection, parent flock age and egg storage and the benefits or impacts of these factors on embryonic development and survival are reviewed. In addition, the mechanisms of embryonic metabolism, cell death and expression of some genes that initiate and execute cell death or affect embryonic development are described. Chapter 2 contains a detailed description of the materials and methods used throughout this thesis project. In chapter 3 the description and findings of using a custom made embryo calorimetry system and its accuracy to measure avian respiratory quotient (RQ), O_2 consumption and carbon dioxide (CO_2) production are provided. In chapter 4 of the thesis a biological application of the calorimetry system to examine the impact of genetic selection and parent flock age on embryonic metabolism in turkey eggs obtained from different genetic

strains and parent flock ages is explained. Chapter 5 highlights the blastoderm dissociation technique developed in this thesis. In this chapter, the technique developed is also used to examine the impact of egg storage on embryonic cell viability in layer eggs. Additionally, in this chapter some procedures used during flow cytometry evaluation of apoptosis and necrosis of stored eggs are outlined. The chapter 6 focuses on the sequence of events such as changes in gene expression, blastodermal cell viability and embryonic metabolism resulting from prolonged cold storage of broiler eggs. The final part of the thesis is Chapter 7. This chapter provides a general discussion of key findings in the thesis. It also shows some future guidelines and how the study outcomes have contributed new knowledge into the field of poultry science. It also discusses how industry can benefit from better eggs storage or to incubate eggs in ways that will improve embryo quality and subsequently hatch quality.

2. OBJECTIVES OF THE THESIS

The overall goal of the thesis was to examine the effects of genetic selection, parent flock age on embryonic metabolism, and the effects of egg storage duration on the whole embryo during and after storage at the cellular, molecular and physiological levels (embryo metabolism). The specific objectives of the current study were in three fold:

1. To determine the effects of genetic strain and parent flock age on eggshell conductance (G), embryonic metabolism and respiratory quotient (RQ).

2. To determine the rate of apoptosis and necrosis in layer and broiler breeder eggs stored for 4 d and 14 d.
3. And to determine if storage of broiler breeder and turkey eggs for 4 d versus 14 d changes the expression level of apoptotic genes.

3. LITERATURE REVIEW

3.1. Avian embryonic development

In the hen's reproductive tract the left gonad and left genital duct develop into the ovary and oviduct respectively while the right organs become arrested during development. Thus, the chicken has only one functional ovary and one oviduct. The yolk of the egg, the ovum and the blastodisc (the fertilized ovum) are formed in the ovary whereas the egg white or albumen, shell membranes and eggshell are formed in the oviduct (Conrad and Scott, 1938). Typically the oviduct is divided into five distinct regions where egg formation occurs. These are infundibulum, magnum, isthmus, shell gland (or uterus), and vagina.

3.1.1. Egg formation and fertilization

The egg is the "container" where the embryo spends its entire life until it hatches. When the hen reaches sexual maturity, yolk formation begins as a gradual deposition of continuous layer of yolk materials in the follicular sac and at the same time embryo formation starts (Romanoff, 1960). Marza and Marza (1935) divided oogenesis, formation of the embryonic cells in the hen into three phases. The first stage is a quiescent period in which the primordial yolk is laid

down and maintained during the formation of the embryo in the ovary. The second period is when white yolk accumulates slowly. This period lasts about 2 months and then merges into the final phase, which lasts for 5 to 9 d prior to ovulation. During the last period yellow yolk is also rapidly deposited from the liver into the follicular sac. Conrad and Scott (1938) reported that yellow yolk formation occurs as continuous, concentric layers except in the region of the blastoderm called the animal pole, or germ spot, where no yellow yolk is deposited. Since the blastoderm remains on the surface of the ovum, a column of latebra or the white yolk forming in the early stages of yolk formation connects the blastoderm with the main bulk of the latebra in the center of the ovum. Normally more yellow yolk is deposited in the opposite hemisphere of the blastoderm (vegetal pole) than in the hemisphere containing the blastoderm (animal pole). This causes the latebra mass to be displaced slightly from the center of the yolk and accounts for the floatation of the yolk in the albumen. This also causes the blastoderm to always stay on top of the yolk surface (Conrad and Scott, 1938).

The formation of the yolk is very important because during incubation the embryo obtains the energy needed for its development mainly by ß-oxidation of yolk fats (King, 2007). When the yolk follicle reaches maturity, it is ovulated and captured by the infundibulum of the oviduct. In the infundibulum, sperm, if present may fertilize the blastodisc. If fertilization should occur, it must proceed before the outer layer of the vitelline membrane (or the outer perivitelline layer) is formed. This is because spermatozoa are not able to penetrate this layer to bind

the sperm receptors in the inner layer of the vitelline membrane (Hrabia et al., 2003).

After fertilization, the yolk moves into the magnum section of the oviduct where the dense portion of the albumen is added. When an egg is laid, albumen can be observed as 4 white layers whose biological function have not been explained very well (Romanoff, 1943). The first of these layers is the gelatinous inner thick or chalaziferous layer, which extend towards the poles of the egg to form the chalazae or the white cord-like structures that hold the yolk in position. The second layer is the inner thin white, composed of 5 to 10 cm of fluid. The third layer is the outer thick or "viscous" white, consisting of about 20 cm of gelatinous material while the last layer is an outer thin white layer of fluid (Conrad and Scott, 1938; Romanoff, 1943). The albumen solid consists mainly of proteins (about 10%) dissolved in water as well as antibacteria properties which provide additional nutrition and protection for the development of the embryo. The albumen also acts like a cushion for the embryo against frequent turbulence (Mickey et al., 1998). The major proteins that make up the albumen are ovalbumin, conalbumin, ovomucoid and lysozyme. These are all synthesized by tubular cells in the magnum mucosa. Mucin forms approximately 2% of the total albumen and these come from certain cells that line the lumen epithelium of the magnum. Other proteins such as immunoglobulin provide immunological functions to the embryo. This protein forms just a small proportion of the albumen. When the yolk passes through the magnum, extensive tubular cells arranged in spiral chords compress the mucosa walls aiding in the release of

albumen components (Moran, 1987). Conrad and Phillips (1938) reported that the inner thin and chalaziferous layers, as well as the chalazae, arise simultaneously by the mechanical segregation of its mucin fibres. Edwards et al. (1976) observed that the concentration of each major albumen protein changes in parallel with magnum weight and during the ovulatory cycle. Unlike the egg yolk, the albumen contains little fat to provide major nutrition for the embryo (Latour et al., 1998).

The next section of the oviduct is the isthmus and its diameter is smaller than the magnum. The inner and outer shell membranes which aid in respiration or gas exchange are formed in this region. The eggshell itself is laid down from the shell gland or the uterus. The avian eggshell is a composite of bioceramic materials formed by controlled interaction between organic and inorganic matter. The organic material contains among other constituents, type X collagen and proteoglycans, mainly keratan and dermatan sulfate. The inorganic portion which forms the bulk of the shell is composed mainly of calcium carbonate (Fernandez et al., 1997). In the uterus the egg stay there for about 20 h for the eggshell to form. The quality of the eggshell is of primary concern to the poultry industry. Successful development of the embryo is dependent upon a robust eggshell for mechanical protection, protection from infection, prevention of excessive water loss, and as a primary source of calcium for development of embryonic skeleton. The last portion of the oviduct is the vagina where the thin protein coating called the cuticle is applied to the outside of the shell. The cuticle helps to keep bacteria from entering the eggshell pores (Smith, 1914). The egg passes through the oviduct small end first, but is laid large end first. In the vagina, the egg is turned

horizontally just before laying and pushed out of the oviduct in a process called "oviposition" (Latour et al., 1998).

After 24 h the egg is finally laid (oviposition). As the egg is laid and cools down from the hen's body temperature (41^{o}C) to the environmental temperature, the inner shell membrane contracts from outer shell membrane at the large end of the egg. This process results in the formation of an air filled space between the inner and outer shell membranes called the air cell or space. The formation of the avian egg as described above is largely derived from the domestic fowl. Presumably, the principles established also apply to all species of birds (Asmundson, 1939). Eggs of all avian species are alike in certain respects, but between chicken and turkey difference are due to egg size and relative weight of the shell membrane (Asmundson, 1939).

3.1.2. Mechanism of ovulation, oviposition and eggs sequence

Ovulation is the release of ovum from the ovary or ovarian follicle into the fallopian tube. In birds the ovulatory cycle is associated with physiological, endocrinological and environmental mechanisms involving a complex interplay of light stimulus, hormones from the hypothalamus, pituitary, gonads, thyroid and adrenal glands (Cunningham et al., 1984). Light stimulation of a mature hypothalamus through the skull results in the release of gonadotrophin releasing hormone (GnRH) into the blood. The GnRH then stimulates the anterior pituitary gland to release gonadotrophin hormones. There are two gonadotrophin hormones: the follicle stimulating hormone (FSH) and luteinizing hormone

(LH). The LH is responsible for sexual maturity and daily release of a mature ovum or egg. As the name suggests FSH is involved in the maturation of tiny follicles into large yellow follicles before they can be ovulated (Robinson et al., 2003).

About 6 h before the hen begins to ovulate it experiences a surge of GnRH, which triggers the release of LH into the blood. The release of LH is limited only to a certain period of the day called 'open period'. Beyond this period the hen will not lay an egg but will defer the next egg to the next day when it establishes another open period. During the release of LH if a mature follicle is present it will respond to the surge of LH and produces progesterone (P4) which stimulates the secretion of more LH into the blood stream. This feed-back mechanism ensures that the maturation of the next follicle which is monitored by FSH and the release of the next follicle due to LH surge occur in the open period. The end point of these hormonal controlled activities is the release of the ovum, called ovulation. The released ovum then enters the infundibulum and egg formation continues through the rest of the oviduct until the egg is laid.

During egg production a hen lays an egg a day and continues to lay an egg for certain period of time. It takes a break of one or few days. Such a series of eggs laid on successive days has been termed as egg sequence. The release of GnRH from the hypothalamus results in egg laying sequence (Robinson et al., 2003). During each sequence the first egg is called the first-in-sequence (C1) while the last egg is called the late laid terminal-in-sequence (Ct). A small daily surge of LH occurring at the onset of darkness ensures that oviposition of one egg

and ovulation of another ovum is restricted to certain periods of the day or the open period (normally 8-10 h period of the day). As the hen ages, the interval between ovulation increases and this results in shorter sequences. First of sequence eggs normally have lower hatchability and are normally bigger because of the greater length of time spent by the yolk in the hen's reproductive tract. Such eggs have more advance blastoderms, larger yolk, albumen and greater percentage of shell compare to subsequent eggs in the sequence.

3.1.3. Major features of embryonic development prior to oviposition

Embryonic development starts at the same time as egg formation begins. In their documentary about development of poultry embryo, Eyal-Giladi and Kochav (1976) divided the development of the chicken embryo during the time of egg formation and soon after oviposition (cleavage to hypoblast) into 14 stages. The first 10 stages (I to X) occur inside the hen during which the egg is being formed. The other four stages, XI to XIV occur after the egg is laid and incubated at an appropriate temperature. Some researchers believe that until the egg finds favorable temperature further development of the embryo is arrested (Fasenko, 2007).

The cleavage stage refers to an embryo (blastoderm) in its earliest stage of cell division. This period lasts from the first mitotic cell division of the fertilized ovum to the formation of the morula (a compact mass of blastomeres). Watt et al. (1993) reported that the morula stage is normally completed by the end of Eyal-Giladi and Kochav (1976) stage X. In their classification, Eyal-Giladi and Kochav

(1976) placed embryos whose eggs had just entered the uterus or had been there for about an hour into stage I. At this stage, the blastoderm is about 3.5 to 4.0 mm in diameter and appears like a disc (Edwards, 1902). The authors called this disc the blastodisc. This stage is characterized by several cleavage furrows with nearly all cells remaining open at their periphery (Eyal-Giladi and Kochav, 1976). The first cleavage furrow in the central portion of the blastodisc is followed by several other cleavages that lead to the creation of a single-layered blastoderm. By stage V some of the features observable in the embryo included a series of closed, bead-like blastomeres that occupied equally large areas on both dorsal and ventral surfaces of the blastoderm. At this stage a sub-blastodermic cavity (Bakst et al., 1997) or sub-germinal cavity (Eyal-Giladi and Kochav, 1976) is established. This cavity is normally formed at stage IV between the blastoderm and the yolk as a result of cell shedding from the ventral surface of the blastoderm to the yolk surface.

At stages V the upper surface of the germinal disc is not smooth. Watt et al. (1993) observed that at this stage the place where the future area pellucida will form was only 3 to 4 cell layers. As the embryo proceeds to stage VI the first morphogenetic phenomenon during embryonic development occurs. This is the formation of one-cell layer called area pellucida as result of the thinning of the ventral surface of the blastoderm. This is mainly due to extra cell shedding similar to what was observed in stage IV. When the embryo is harvested, the shed cells are usually observed resting on the yolk surface at the lower face of the subgerminal cavity as white particles. The same morphogenetic process that leads

to the placement of the area pellucida also establishes the anterior-posterior axis of the embryo (Patten and Calson, 1974). During the stages between VI and X there is more shedding of cells leading to the distinct appearance of the area pellucida and the area opaca (the peripheral opaque area that surrounds the area pellucida).

In the Eyal-Giladi and Kochav (1976) stage X embryo, occurring after the egg is oviposited the blastoderm is seen as a flat compacted disc, about 4.4.mm (Edwards, 1902) or 4 mm in diameter (Zagris et al., 1998; Bakst et al., 1997). In addition, the area pellucida is completely formed and clearly delineated from the area opaca. Physically, the area pellucida develops into the embryo while the area opaca forms the extrembryonic structures or primordium of the yolk sac (Bellairs et al., 1967). The initial indications of hypoblast formation (development of blastoderm into embryo) called gastrulation is only observable after stage X as clusters of small cells in the area pellucida (Figure 1.1).

Gastrulation in the embryo begins with the formation of a primitive streak through which precursors of definitive mesoderm and endoderm ingress and migrates to their embryonic destinations. This organization is primarily induced by signals from the posterior margin of the blastodisc (Wei and Mikawa, 2000). The period of gastrulation or the final period of early morphogenetic development, also hypoblast formation has been associated with Eyal-Giladi and Kochav (1976) stages XI to XIV. These stages can only advance upon incubation of the egg and provision of favorable incubation environment (Patten and Calson, 1974).

3.1.4. Events marking embryonic development after oviposition

The period of embryonic development regarded as late embryonic stages extends from some hours after egg laying (Eyal-Giladi and Kochav (1976) stage XVI or the Hamburger and Hamilton (1951) stage 1 (HH1)) until the egg is hatched (HH46). The earliest parts of the Hamburger and Hamilton (1951) classification have been shown to overlap with the Eyal-Giladi and Kochav, (1976) stages XI to XIV. During this period blastodermal cells differentiate and increase in number by mitosis. Baggot et al. (2001) observed that by 44 h after incubation the heart and vascular systems have joined together while the heart starts to beat. At this time two distinct circulatory systems are established; an embryonic system for the embryo and a vitelline system extending into the egg yolk which is used to derive yolk nutrients. Maturity of both the respiratory and cardiovascular systems is crucial to ensure efficient delivery of O_2 to support metabolic heat production and regulate body temperature (Black and Burggren, 2004).

The development of the extra-embryonic membranes occurs as temporal appendages and persists until the embryo can fully use its lungs. These appendages consist of the yolk sac, amnion, chorion, and allantois. The chorion and allantois fuse together to form the chorioallantois, an extension which encloses the albumen (Romanoff, 1960). The yolk sac is formed as an outer area of the blastoderm and it consists of three cell layers, which are in continuation with the cell layers of the embryonic disc. These are an ectodermal layer adjacent to the vitelline membrane, an endodermal layer adjacent to the yolk and

in between them is a layer of mesoderm cells. The yolk sac (which persists as the viteline membrane until 4 d of incubation) surrounds the yolk. The yolk sac membrane passes the equator of the yolk sac at 5-7 d and achieves its maximum area around 10-11 d finally surrounding yolk at 14-15 d. Its highly vascularized portion extends to receive absorbed nutrients from the yolk and transfers them to the embryo. By the end of 4 d of incubation all the organs needed to sustain embryonic life are formed (Patten and Calson, 1974).

The amnion is formed from a layer of the endoderm underlying the avascular (does not develop blood vessels) mesoderm immediately close to the embryo. The appearance of muscles in the mesoderm allows the amnion to become contractile by 5 d of incubation. Blood flow through the amnion is supported by the fusion of the avascular vessels with the vascular mesoderm from the allantois. The formation of a duct between the albumen and the amniotic sac called the seramniotic connection allows the movement of albumen protein into the amniotic fluid and subsequently into the yolk sac (Baggot et al., 2001).

By 7 d, digits appear on the wings and feet, and the heart is completely enclosed in the thoracic cavity. Because major organ development usually occurs in the first third of incubation (1-7 d), unfavorable conditions such as low or high temperatures and heat stress could trigger malformations. The Chorion develops from a tissue continuous with the amnion and away from the embryo. The allantois grows as a small bud from the primitive hindgut of the embryo and is also vascularized. By 11 d of incubation, the allantois completely lines the inner portion of the chorion. The resulting choriollantois which forms gradually

increases to cover about 98% of the area of the eggshell membrane by the time the embryo prepares to hatch. The fusion acts as the primary respiratory surface especially during the second period of incubation (Baggot et al., 2001).

The embryo grows and develops rapidly during the first third of incubation through cell division and differentiation of vital organs. At this point it depends largely on carbohydrates in the yolk to obtain energy. The energy source being utilized by the embryo can be established by obtaining the respiratory quotient (RQ). In the initial period of incubation the RQ value is close to 1, which means the embryo is depending largely on carbohydrates and in the latter parts of incubation the RQ drops close to 0.6 which indicates that the embryos is metabolizing mostly lipids (Hamidu et al., 2007). During the second period of incubation (8 to 14 d) in chickens the embryo grows in size. Between 4 to 8 d of incubation the metabolism of embryos is relatively low (1 to 4 mW), but by 14 d of incubation it has risen steeply to about 121 mW of heat production with O_2 consumption reaching almost 500 ml/d (Hamidu et al., 2007). After 10 d of incubation, feathers and feather tracts are visible, and the beak hardens. This occurs 12 d onwards in turkey embryos (personal observation). In the last third of incubation (15 to 19 d in chickens and 20 to 26 d in turkeys) more body mass is added.

At about 18 and 19 d (chicken) and 24 to 26 d (turkey) the embryo breaks into the air cell (at the blunt end of the egg) in a process called internal pipping. The presence of egg tooth attached to the beak of the chick assists greatly in breaking through the eggshell membrane. After this point the embryo draws in O_2

reserve in the air space to inflate its lungs to supplement the supply of O_2 by simple diffusion through the eggshell pores. From this point there is a gradual shift of the embryo from using the chorioallantoic membrane for respiration to pulmonary respiration. During the process of internal pipping, the embryo uses stored carbohydrate for energy rather than fats which require more O_2 during ß-oxidation (Miller, 1998). As soon as the embryo pips into the air cell, it uses the drawn in O_2 to help provide the energy needed for the next stage of pipping out of the eggshell. In some embryos this O_2 may still not be sufficient to pass the embryo through the more difficult stage of breaking through the eggshell (external pipping) due to lowered O_2 partial pressure or tension (differential assess through the shell). At 20 d (chicken) and 27 d (turkey) of incubation, the embryo is in hatching position. With the head under the left wing it uses the egg tooth and beak to externally pip through the eggshell. By this point the embryo is almost exclusively using the lungs for respiration. After externally pipping out of the shell, the chorioallantois, which had served as the primary embryonic lungs during the early and second part of incubation, dries out. The remaining yolk sac is drawn into the abdominal cavity of the embryo (Smith, 1914). On hatching the chick or poult becomes exhausted and rests, while the navel opening heals and the down feathers dry.

3.1.5. Developmental comparison of chicken and turkey blastoderms

Morphologically, there is only little stage by stage comparison between the chicken and turkey blastoderms (Eyal-Giladi and Kochav, 1976; Gupta and

Bakst, 1993). The number of blastomeres present from the stages I to IV are normally higher in turkey embryos than in chicken embryos (Bakst et al., 1997). However, this comparison of the two blastododerms was obtained from specimens where the turkey embryo was normally 1-2 h older than the chicken embryo. Therefore there is a difficulty to make a strict developmental comparison between chicken and turkey embryos growing from the cleavage to hypoblast stages. Nevertheless, while Bakst et al. (1997) noticed that in chickens the stage IV coincided with the formation of only the area opaca, in turkeys a small irregular eye called the area alba also appeared in the middle of the cells in addition to the area opaca. In both chicken and turkey the appearance of the area pellucida marked the beginning of stage VII and becomes prominent as result of continuous division of the blastodermal cells found at the dorsal region of the blastoderm. In turkeys the egg is ready for oviposition at stage VII, but in the chicken the blastoderm progresses to stage X before oviposition. These developmental differences could account for differences in blastodermal cell numbers in freshly oviposited eggs in chickens: 60,000 cells (Petitte et al., 1990; Gupta and Bakst, 1993; Etches et al., 1996) and 32,000 in the turkey blastoderm (Bakst and Akuffo, 1999). The limited development of the turkey embryo as well as the relatively lower final blastodermal cell numbers at oviposition could account for the longer incubation time of turkey embryos compared to chicken embryos. Sellier et al. (2006) realized that irrespective of the developmental differences it took about the same time (23 to 26 h) for turkey and chicken to reach the end of blastoderm development, which is at stage VII and X

respectively. This implies that under normal circumstances, turkey embryos will require extra time to develop to stage XIV during which primitive streak formation will begin as compared to chicken embryos. In chickens, Bakst et al. (1997) noted that by stage XIV, the anterior aspect of the hypoblast was well defined while the posterior aspect of the hypoblast and the area opaca formed a cellular bridge in an event immediately preceding primitive steak formation; however this is not the case in turkey eggs.

3.2. Eggshell conductance

Eggshell conductance (G) is the ability of gases and moisture to diffuse across the eggshell and the chorioallantoic capillary blood which is subsequently utilized by the embryo for respiration (Chiba et al., 2002). Onagbesan et al. (2007) in a review have cited other studies that reported evidence that it is the G that determines the precise amount of O_2, CO_2 and water vapour that is exchanged between the internal and external environment of the egg. Christensen et al. (2006b) reported that the G is a component of a multifactor relationship involving egg weight, incubation length and eggshell conductance constant. Other factors that have been used to express G include eggshell thickness (Ar et al., 1974; Peebles and Brake, 1987) and specific gravity of the egg (Hamilton, 1982). Tullet and Noble (1989) showed that for accurate assessment of G, pore formation, shell thickness, total functional pore area, and the resistance to the passage of gases and water vapor through the eggshell pores should be taken into account. Thus, diffusive conductance of gases in and out of the egg increases if eggshell pores

are more numerous, wider and shorter (Onagbesan et al., 2007). Typically the pore numbers in domestic hen range between 7,000 and 17,000 (Solomon, 1991).

Gas exchange in avian embryos has been shown to be dependent on, and limited by, the diffusive properties of gases across the resistance offered by the eggshell and the eggshell membranes (Wangensteen and Rahn 1971). This resistance is dependent on the G and egg size (Hamidu et al., 2007). Therefore an accurate assessment of G will help to incubate eggs properly to ensure better eggshell function. While there is no one method to assess the ability of the eggshell to support embryonic life, in the study conducted by Ar et al. (1974) they set up a standard that was used to describe G. This standard expresses G (G_{H_2O}) as the ratio of the rate of moisture loss from the egg (M_{H_2O}) to the change in saturated vapor pressure of the egg and the outside environment (ΔP_{H_2O}). Where, G_{H_2O} = water vapor conductance or eggshell conductance (mg/day/mmHg); M_{H_2O} = the rate of water loss from egg (mg/day); $\Delta P_{H_2O} = P_{H_2O} - P_o$; P_{H_2O} = water vapor pressure of the egg; P_O = water pressure outside the shell; and ΔP_{H_2O} = water vapor pressure difference across the shell, which, is equal to the saturated vapor pressure (mmHg). With this expression it is expected that larger eggs will have higher G value compared to smaller eggs.

3.2.1. Eggshell conductance affects embryo respiration and development

The avian embryo exchanges O_2 and CO_2 by simple diffusion through the eggshell pores. The respiration of an incubating egg is performed by the yolk sac

during early periods of development. But as the embryo grows the chorioallantois (fusion between the chorian and allantois membranes) replaces the yolk sac until pulmonary respiration takes over by the time the embryo is ready to hatch. The outer limb of these extra-embryonic membranes is well vascularized to support the uptake of O_2 from the air. In the later stages of development the chorioallantoic capillary extends over the entire inner surface of the shell (Tawaza, 1980). Between the ambient air and the capillary blood the respiratory gases are exchanged by diffusion through the porous shell. While some researches focused on the whole eggshell as gas resistance medium and sometimes just the calciferous shell, such studies have not shown that the two fibrous shell membranes (inner and outer shell membranes) and the chorioendothelium are the main resistance to gas flux. Even though the resistance to gas diffusion (defined as the reciprocal of G) for the outer barrier is almost fixed throughout incubation, that of the inner barrier decreases as the embryo develops as result of the eggshell membranes degenerating (Tazawa, 1980). Because G is fixed from the outer barrier (same egg shell pores), at some point during incubation the embryo is obliged to take up O_2 under hypoxic conditions even though its metabolism may be expected to be higher (Dawes and Simkiss, 1971). At the same time the respiratory factors of the allantoic circulation (e.g. blood vessel thickness) changes progressively with embryo development to moderate constrains on gas exchange through the eggshell. When a hypoxic state is created the embryo relies increasing on increased allantoic blood flow and increased chorioallantoic capillary volume to increase G of the inner barrier (Tazawa, 1980).

It is well known that hyperoxia stimulates growth late in the incubation period. This occurs until the embryo outgrows the O_2 diffusion capacity at the plateau stage of O_2 consumption (Christensen et al., 1997). The authors showed that at the plateau stage of O_2 consumption, turkey embryos from genetically selected lines had lower G value than those from random bred lines. However, this resulted in higher late embryo mortality in the selected strains (Christensen et al., 1993). Christensen et al. (1997) suggested that a lower G value may have resulted in greater hypoxia that depressed the metabolism of the embryos from selected lines. Moreover, when the selected lines were supplemented with O_2 the poults had heavier hearts and body weights than those from the control groups indicating that the embryos were efficiently taking up "pure O_2 compared to if selective respiration of O_2 from ordinary air'. The increased performance after supplementation could result from the abundance of O_2 molecules available to the embryo even though eggshell conductance remained the same. In other words, better performance could also be due to the entrance of undiluted O_2 which did not have to compete with other gases to passively diffuse into the eggs. Van Golde et al. (1998) wondered if hypoxia can also affect embryonic development during the early period of incubation. In this study, they exposed three groups of chicken eggs to 60% O_2 during 10, 14 and 18 d of incubation and obtained embryo and organ masses (brain, heart, lungs, liver and intestine) 24 h after the start of hypoxic exposure until d 21 of incubation. In this experiment, they observed that in all organs, intestines had the highest rate of growth. Since the growth of highly O_2 demanding organs were limiting during these periods, they

concluded that O_2 availability can be a limiting factor for growth even before metabolism exceeds the O_2 diffusion capacity of the eggshell in later part of incubation. At this point eggshell conductance may not be a limiting factor for gas exchange but the partial pressure of O_2 was the limiting factor to embryonic development.

3.2.2. Relationship between eggshell conductance and water loss

One of the most important factors determining hatchability of avian eggs is proper water balance. In turkey eggs, a total diffusive water loss of 12 ± 1% of initial egg mass over 28 d of incubation was shown to yield the maximal hatchability irrespective of the combination of G and incubator relative humidity (RH) which brought about this water loss (Meir et al., 1984). A good correlation was also found between G at the beginning of incubation and the final total diffusive water loss for a given RH. However, when eggs were sorted into low, medium, and high G categories and matched with the appropriate RH, hatchability increased (Meir et al., 1984). The authors noted that poor hatchability was only obtained in mismatching RH and G because the value of G can determines how much moisture can be lost. It seemed that hatchability success may be experimentally improved if a correct rate of water loss is fitted to sorted eggs during incubation. In a later trial, Meir and Ar (1987) also suggested that a proper water budget can be used to increase both hatchability and poult quality.

By physically covering a portion of turkey eggs with paraffin wax to reduce G (a possible experiment to measure effect of moisture loss on embryo

performance), Christensen et al. (2006b) measured the mean heart rate of embryos in each of three G categories. Their observations showed that decreasing G had direct effect on embryonic heart rate. The embryonic mortality rate was higher in high G eggs with accelerated heart rates than those in low G eggs with reduced heart rates. However, the authors argued that increased porosity and increased heart rates seem contradictory because embryos in eggs with low G would have a greater need for increased tissue oxygenation and therefore heart rates. They believed that embryos from higher G eggs die from more rapid heart rates which induce cardiac failure. The embryos developing in low G eggs had an advantage because of their heavier heart which enabled them to pump more blood without using any extra energy. In addition they had heavier myocardium and showed better energy balance with more glycogen than lactate to be used for muscular activity compared to embryos in high G value eggs. From the authors own observation, these results conflict with those from earlier study, where Christensen et al. (2006a) reported that more embryos from eggs of high or average G survived better than low G eggs. However, in their earlier study the authors reported that when poults from high G eggs were placed they did not grow well compared to poults from average or low G eggs. Since hatchability at the end of incubation and broiler final body weight in both studies were not provided further investigations on the G value is needed to determine its impact on embryo survival and posthatch performance.

3.3. Embryonic metabolism

Metabolism is the sum total of all the biochemical reactions occurring in the body of an organism and the release of energy. This energy is needed for body maintenance, growth, reproduction and exercise (Krogh and Lindhard, 1920). At the end of metabolism CO_2, water and metabolic heat are produced as by-products. At the cellular level glucose is the primary molecule used in metabolism. When glucose is limiting, the body resorts to the use of either lipids during β-oxidation or proteins or glycerol from lipid during gluconeogenesis. Once glucose is broken down into pyruvic acids, O_2 is used to further breakdown the pyruvic acids in other metabolic pathways (Kreb's cycle, electron transport chain and adenosine triphosphate or ATP cycle) to release the bulk of the energy required by the organism (Vieira, 2007).

During incubation avian embryos obtain O_2 by simple diffusion through the eggshell pores. The O_2 is used to metabolize yolk nutrients and in the process CO_2 is release as by-product through the eggshell pores. In all precocial birds such as chickens and turkeys, the embryo begins to show significant growth after 4 d of incubation at which point the heart beat is very significant. This period also coincides with the period of active O_2 consumption (Rahn et al., 1979). From 4 d of incubation onwards O_2 consumption increases exponentially as the embryo grows and reaches a plateau at about 80% of incubation (Dietz et al., 1998). The plateau stage means that the O_2 consumption, CO_2 production and embryonic metabolism remain the same for some days even though the embryos continue to gain weight. In chicken and turkey embryos the plateau stage is reached at 18 d

(Rahn et al., 1979) and 25-26 d (Christensen et al., 2004a) of incubation respectively. At the plateau stage of O_2 consumption embryonic growth is higher than O_2 consumption. This creates a hypoxic state that stimulates several physiological functions leading to pipping and hatching of the embryo. Vleck et al. (1980) reported that if embryonic growth continues during and after the plateau phase while embryonic metabolic rate stays the same, there is an energetic paradox. This means that increasing embryo mass would increase the cost of energy that will be used for body maintenance and egg metabolism (Dietz et al., 1998). However, this energy may not be available because respiration and metabolism slows down.

In avian embryos, the daily energy expenditure of an egg can be divided into five components: (1) embryonic maintenance or the metabolic costs to maintain the embryonic tissue in a steady state, (2) synthesis costs, that is, the energy spend in depositing new embryonic tissues and not energy content of the deposited tissues (3) muscular activity, particularly during hatching process, (4) energy for growth and metabolism of extra embryonic membranes, which are relatively large early during incubation, and (5) energy deposited as new embryonic tissues or energy of the deposited tissues. With the exception of the energy of the deposited tissues, all the other energy groups are difficult to measure (Dietz et al., 1998). The energy of the deposited tissues is the actual energy of embryonic metabolism, and can be measured by using the O_2 consumption of the embryo. Since embryo growth and metabolic rates increase daily there is the need to investigate this daily energy of metabolism to explain

embryonic growth pattern. The authors acknowledged that it is difficult to estimate differences between growth and maintenance components of embryonic metabolism during development. Hence most energy calculations of an avian embryo during incubation are based on the two components (Dietz et al., 1998).

3.3.1. Pattern of embryonic metabolism during incubation

As exemplified in the chicken embryos, O_2 is obtained through the eggshell pores until external pipping (Patten, 1951). During the initial period of incubation, the embryonic O_2 consumption is very small until about 4 d of incubation (Rahn et al., 1979). For the first 2 d of incubation, the embryo respires using the yolk membrane (sac) until the chorion and the allantois membranes fuse together to form the chorioallantoic membrane around 4 to 5 d of incubation (Onagbesan et al., 2007). Following this fusion O_2 is absorbed by the choriollantoic membrane before it reaches the embryo. Carbon dioxide also leaves the embryo through the same respiratory pathways. The chorioallantoic membrane persists as the main respiratory organ until the embryo begins to partially use its lungs for respiration (after internal pipping) and fully after external pipping (Rahn et al., 1979).

From 4 d of incubation until O_2 consumption reaches a plateau, embryonic O_2 consumption and metabolic rates increase exponentially (Rahn et al., 1979). Onagbesan et al. (2007) reported that during the plateau stage, the gas fluxes between the embryo and the environment depend on gas partial pressures in the blood of the extra-embryonic circulation, the effective gas exchange area,

thickness and diffusive properties of the material separating the red blood cells from the environment. It has also been noted that during the plateau stage of O_2 consumption the O_2 requirement of the developing embryo exceeds the diffusion characteristics of the eggshell (Christensen et al., 2001c). The plateau stage of O_2 consumption is created because embryonic growth is higher than the rate at which O_2 can diffuse through the eggshell pores by simple diffusion (these are fixed during egg formation). Research has reported that the plateau stage creates hypoxia, hypercapnia and creates the paradox of embryonic growth and embryo metabolism as explained earlier (Christensen et al., 2004b). Prior to the plateau stage, vital tissues such as liver and muscles accumulate glycogen to fuel embryonic survival through the plateau stage (Christensen et al., 2004b). This is because glucose requires less O_2 to provide the needed energy to the embryo compared to the usual yolk lipids that the embryo uses for most part of incubation. As plasma glucose concentration declines at the plateau stage, CO_2 concentration increases (hypercapnia) and this is believed to trigger the pipping and hatching process of the embryo (Visschedijk, 1968). In the beginning of hatching, the embryo first internally pips into the air cell at the broad or blunt end of the egg. Because this region contains air rich with O_2 the embryo breaths in O_2 for the first time to inflate its lungs. From this point the embryo uses both the chorioallantois and its lungs for respiration (Patten, 1951). As pulmonary respiration increases, metabolic rates also increase (Hamidu et al., 2007). Gradually, the chorioallantois degenerates and the embryo breaths fully with its lungs.

3.3.2. Measuring embryonic metabolism

Measurements of avian fetal heat production or metabolism involve putting the eggs in metabolic chambers and measuring O_2 consumption and CO_2 production rates (Romijn and Lokhorst, 1960; Hamidu et al., 2007). Many studies that measured avian embryonic O_2 consumption, CO_2 production and metabolism in the past have used chicken embryos and very simple techniques (Vleck et al., 1980; Monge et al., 2000; O'Dea et al., 2004; Mortola and Labbe, 2005). This is because the chicken egg is a good experimental model for avian respiratory studies. However, the only record of O_2 and CO_2 measurements extensively described for turkey embryos was reported by Dietz et al. (1998).

They initially measured metabolic rates on pairs of eggs until 11 d of incubation. But from 11 d onward individual eggs were used. The use of pairs of eggs can make it difficult to accurately assess individual embryo performance and gas exchange. A total of 20 metabolic chambers were used and each of 0.4L chambers was ventilated with ambient air. The chamber temperature was equivalent to the incubation temperature and air flow rates were adjusted by a mass flow meter set at 500 ml/min. The flow meter was also used to maintain O_2 and CO_2 concentrations of the dried outlet air. After the eggs were put into the metabolic chambers, the system was allowed to reach equilibrium during a period of at least 60 min. The actual measurements took an average 15 min per chamber. The O_2 and CO_2 concentrations of the inlet air were however measured hourly to ensure a study flow of dry air into the chambers. The O_2 and CO_2 concentrations were continuously measured from the dried outlet air from each chamber. One

disadvantage about this system was that both O_2 and CO_2 analyzers were not very sensitive and required longer time to register stable concentrations of O_2 and CO_2 gases. Because the longer wait time could ignore environmental fluctuations it is important that such measurements are taken over a range of time and an average value utilized. The average egg metabolic rate (M egg; mW) was computed as: M egg = 4.49 V O_2 + 1.39 V CO_2 , where V O_2 was the volume of O_2 consumed and V CO_2 was the volume of CO_2 produced. The methodology utilized is however similar to the calorimetry system recently used in another to describe respiratory and energy metabolism between broiler breeders of different genetic strains and parent flock ages (Hamidu et al., 2007). However, the constants used to obtain the embryonic metabolic rates were based on formula derived by Kleiber in 1987. With incubation efficiency of turkey eggs recently receiving some attention as broiler eggs, it is important to investigate to what extent genetic selection and maternal age has also impacted the embryo's metabolism using some of these systems that are able to provide embryonic respiration in both directions (i.e. O_2 and CO_2 exchange).

3.4. Genetic selection

In turkeys, selection for meat yield has caused the male to grow to 19.6 kg at 21 wk with feed conversion ratio as low as 2.63. Consequently, it yields 27.6% of live weight as breast meat and over 50% of the live weight as meat (Mckay et al., 2000). However, the genetic progress made has also increased embryonic mortality, reduced embryo heart weight in proportion to embryo size (Bagley and

Christensen, 1989) and this has changed metabolic functions (Christensen et al., 2003). Bagley and Christensen (1989) attributed reduced embryonic heart weight, a condition that increases poult flip-overs to exposing embryos to lowered O_2 tension which is often common in North American altitudes. Some of these conditions are partly due to genetic selection. The authors further showed that in turkey strains selected for growth and breast meat production, extra energy is diverted to the development of tissues such as pectoral muscles in preference to the heart which could increase the chances of metabolic disorders (Bagley and Christensen, 1989). Nestor and Noble (1995) found that embryonic viability has decreased in turkey lines selected for heavier post hatch body weight compared to random bred strains. But Christensen et al. (1993) reported that most of the embryonic mortality normally observed in modern strains occurred during late periods of incubation when a lot of physiological activities occur prior to hatching.

3.4.1. Effects of genetic selection on egg and eggshell properties

Shell thickness mainly provides structural support to the egg, but also plays a vital role in the gas exchange of the embryo (Rahn et al., 1979). Differences in the thickness of the eggshell can be seen among different species or breeds of birds. Since bigger eggs are more likely to have more eggshell pores, species that lay heavier eggs have higher G value (Ar et al., 1974). However, between different modern turkey genetic strains G values do not change (Christensen et al., 2001a). In chickens it was realized that the G value did not

change between strains even where egg weights were varied (O'Dea et al., 2004) or maintained (Hamidu et al., 2007).

Research shows that egg weight has increased about 11% mainly due to the effects of genetic selection for growth. However egg allometric measurements such as egg length and width have not increased at the same rate (Christensen and Nestor, 1994). This is because the weight increase mainly results from yolk and albumen. Just as the sizes of eggs from different turkey strains differ, the compositions of the egg also differ. Research has shown that between Nicholas (N) and British United Turkeys of America (B), eggs from N hens were always heavier due essentially to larger albumen content (Reidy et al., 1998). In a related study, eggs from N strain yielded the smallest yolk weight compared to B strain (Reidy et al., 1994). As part of the impact of genetic selection on all aspects of productively, increase in egg weight can be directly carried over as poult and broiler weight at slaughter. Past research shows that initial egg weight was correlated with late embryo weight (starting from 20 d of incubation), poult weight at hatch, and poult carcass weight (i.e. yolk free body mass) at hatch (Bielfelt and Shultz, 1989). When analysis of covariance was done to remove the effects of egg weight in this experiment, no significant difference was found between embryo weights at 8, 12, 16, 20, and 24 d of incubation and, poult weight at hatch and carcass weight. This is an indication that initial egg weight which can be dependent on genetic strain or breed has a bigger effect on these characteristics. The same study also showed that when N strain was compared to B and Hybrid turkey strains (H), N hens had a smaller percentage of yolk and

greater albumen than H or B hens. However, the mean percentage of shell weight was greatest in H eggs than N or B eggs (Bielfelt and Shultz, 1989). The study appears to suggest that genetic strain also affects eggs components even before eggs are incubated.

3.4.2. Effects of genetic selection on embryo performance

Long term selection for growth by the turkey breeder industry has resulted in major growth and performance changes over the past 40 to 50 years (Christensen et al., 2008). Havenstein et al. (2007) reported that the growth rate of domestic turkeys has more than doubled from 1966 to 2003. On the other hand, the body weight of poults from strains selected for growth has dramatically increased from the body weight of the random bred line from which they were derived (Nestor et al., 1996). Christensen et al. (2008) also reported that in turkey lines selected for growth, embryos hatched at a reduced rate compared with random bred lines. The authors noted that most of the embryos from the modern strains died at the plateau stage of O_2 consumption or immediately preceding pipping and hatching. When random bred lines were back crossed to the genetically pure lines, embryo livability improved modestly due to additive and reciprocal genetic effect. However, the reciprocal genetic effects caused a decrease in embryo heart weight relative to body weight after the plateau stage of O_2 consumption (Christensen et al., 2008). Olsen (1942) found that in turkey eggs the length of the incubation period was 637.6, 639.8, and 641.6 h in eggs weighing 70 to 80, 80 to 90, and 90 to 100 g respectively. Clearly, as egg weight

increases the incubation length also increases. Cherms (1969) found no association between egg weight and length of the incubation period in turkey eggs. But Christensen et al. (2001a) showed that incubation length averaged 662 h between two undisclosed turkey genetic strains that differed in age by less than 1 wk. However, the individual hatched time was not different between genetic strains probably because of the shorter age interval even though, the average initial egg weight differed between strains. Whereas genetic selection for growth in turkeys has resulted in dramatic changes in egg weights, embryonic weight (Nestor and Noble, 1995) and the time taken to hatch (Christensen and Nestor, 1994; Christensen et al., 2000a) no study has made compensative investigation into embryonic metabolism (Christensen et al., 2008).

3.4.3. Effects of genetic selection on embryonic metabolism

Research on factors affecting embryonic metabolism in the past 20 years has paid more attention to chickens compared to turkeys. In chickens, the effects of varying incubation temperature on eggshell temperature and its subsequent effect on embryonic heat production has been established (Lourens et al., 2005). Research has also shown that the adverse effects of elevated incubation temperature on embryo performance do not exist alone (Christensen et al., 2005). Factors such as genetic selection, parent flock age, O_2 concentration and temperature (Christensen et al., 2001a; 2004a, b, 2005; Tona et al., 2004; O'Dea et al., 2004; Hamidu et al., 2007) have been suggested to be key factors also influencing embryo performance. In chickens, elevated O_2 concentration during

incubation was shown to increase embryonic heat production (Lourens et al., 2007). In the event of increased O_2 consumption by the embryos, CO_2 production also increased (Hamidu et al., 2007). Since there is a direct relationship between embryonic heat production, O_2 consumption and CO_2 production, embryos that have the potential to consume more O_2 also have the potential to produce higher CO_2 and embryonic heat (Hamidu et al., 2007).

Christensen et al. (2008) reported that long term selection for increased egg production and 16 wk body weight has altered energy metabolism of embryonic heart as a result of more rapid heart rates. This study also showed that embryos from strains selected for growth had different energy metabolism that relied more on gluconeogenesis. In gluconeogenesis extra O_2 is used to convert end products of glycolysis such as pyruvic acid and lactic acid back to glucose. Subsequently, embryo mortality occurs more frequently in embryos from selected lines when the energy metabolism of the myocardium shows elevated glycogen to lactate ratios. This may be due to inefficient utilization of available glycogen. At the same time the embryo may become weak and die from lack of energy. Christensen et al. (2000b) showed that genetic differences could cause variation in blood glucose levels between embryos from two different turkey strains. The above study was then used to predict that metabolism of other nutrients such as lipids and proteins may also be changing.

Christensen et al. (1999a) realized that in the presence of different O_2 partial pressures, 171 or 152 mm Hg from 25 to 28 d of incubation embryos from strain selected for 16 wk body weight displayed lower lactate and β-

hydroxybutyrate levels compared to their random bred strain. The results mean that embryos from selected strains could not respond to elevated partial pressures of O_2 by adjusting energy metabolism (Christensen et al., 1999a). This inefficiency could increase embryonic mortality during the later periods of incubation. In turkeys, increased blood glucose concentration has been reported as an indication of poor embryonic metabolism (Christensen et al., 2002). This was reported after differences in glucose concentration at the plateau stage of O_2 consumption between N and B strain were observed since glucose utilization is an indication of higher embryonic metabolism.

Oxygenation during pipping and hatching depressed embryonic blood lactate, urates, and β-hydroxybutyrate in genetically selected turkey strains but the same factors were elevated in random bred control embryos (Christensen et al., 1999a). These changes appeared to suggest that embryos from selected lines showed more fluctuation in response to environmental O_2 than embryos from random strains (Christensen et al., 1999b). Other indicators of embryonic metabolism measured by Christensen et al. (2007b) were maltase and alkaline phosphatase activity in the jejunum of turkey embryos. Christensen et al. (2007b) reported significantly lower maltase activity in embryos from genetically pure lines than that in the random breed strains and even in day-old hatchlings and 3-d old poults of the same strains. Similarly, lower levels of alkaline phosphatase activity were reported in genetically pure line. The above observations could indicate a lower metabolic activity of their jejunal tissue and a diversion of this energy to the heart and muscles.

3.5. Parent flock age

3.5.1. Effects of parent flock age on egg and eggshell properties

It is well documented that small eggs hatch sooner than larger eggs and that vital gas exchange and metabolism of embryos from young hens may be compromised. These imbalances in respiratory gas exchange have been attributed to the low G value of small eggs (Ar et al., 1974; Rahn et al., 1979). In turkeys, Christensen et al (2001a) showed that G was directly related to parent flock age. However, this study and many others conducted in chickens (Peebles and Brake, 1987; O'Dea et al., 2004) all recognized that eggs from older parent flocks are bigger than eggs from younger parent flock ages, and so could have higher G values. Fairchild et al. (2002) reported that increased early embryonic mortality in eggs from young parent flocks may be due to decreased G value because such eggs are normally small with limited gas exchange capabilities. For older parent flock ages, embryonic mortality increased during the late period of incubation because embryos from bigger eggs easily outgrow the ability to obtain O_2 by simple diffusion through the eggshell pores. This condition could lead to late incubation hypoxia if pulmonary respiration delays.

Regardless of species, as hen age increases egg size also increases due to increases in yolk deposition (Moran and Reinhart, 1980). In N hens studied from 36 to 55 wk of age the relative yolk weight (DM) increased while relative albumen weight decreased with age. Egg moisture loss through 25 d of incubation was greatest in eggs from 36-wk-old hens compared with hens at 41, 45, 50, or 55 wk. Mean poult weight was also shown to increase from 62.6 g at 36 wk to 64.9 g

at 55 wk. And the relative poult weight when initial egg weight was taken into consideration increased from 36 to 55 wk (Applegate and Lilburn, 1996). Siopes (1995) reported that in all turkey genetic strains, percentage of yolk increases at the expense of albumen as parent flock age increases.

3.5.2. Effects of parent flock age on embryo performance

In turkey production, it is common to experience poor performance from young parent flock ages. However, as parent flocks get older some negative aspects of production such as mortality may also increase to reduce production efficiency. Applegate and Lilburn (1998) reported that even when initial egg weight was maintained between flock ages, a significantly heavier poults weight was observed in 55 wk old hens as against younger hens (36-wk-old). The differences in poult weight were attributed partly to differences in yolk size and yolk sac weight in older hens. Both absolute and relative yolk-free embryo weight at 25 d of incubation have also been shown to increase with age of hen (Applegate et al., 1996). Considering the fact that egg size and egg weight increase with parent flock age, the data also suggest that larger eggs have a higher probability of producing poults with heavier body weight, heavier yolk and solid yolk components.

The effect of hen age on incubation time has been investigated. Considering pooled data from different genetic strains, the time taken for embryos to hatch increased as turkey flock age increased from 33, 43 to 55 wk (Christensen et al., 2001a). Improved embryo livability in turkeys has been linked

with improved yolk utilization, heavier liver and skeletal muscle weight in embryos. However, these improvements lengthened the incubation time in heavier body weight embryos by 10 h when compared to lower body weight embryos (Christensen et al., 2004c). At 33 and 43 wk of age, hatchability of both flocks was higher than 90% but the hatchability decreased to 85% as flock age reached 54 wk which suggests that performance of older flocks could also decrease because of the longer time spent to hatch in the incubator (Christensen et al., 2001a). This can increase overheating and late incubation mortality around pipping and hatching. The lowered hatchability could also be due to more aging blastoderms in eggs of older parent flock ages which spend more time in the reproductive tract before oviposition.

3.5.3. Effects of parent flock age on embryonic metabolism

Researches available on metabolism of turkey embryos have mainly focused on influences of genetic selection (Christensen et al., 1999a; 2007b). Nevertheless, Christensen and Nestor (1994) also stated that difference may exist in the energetics of embryos from hens of different age and these energetic differences could result in poor hatchability and poult quality of some flocks. There is therefore a need to determine effects of selecting eggs from different parent flock ages to determine consumption of O_2, CO_2 production and embryonic heat production and if these could be related to overheating during incubation. This research could be of value to determine if metabolism has any relationship

with higher rates of early embryonic mortality in younger parent flocks as well as increased late embryo mortality in older parent flocks (Reidy et al., 1998).

3.6. Hatching egg storage

Cold storage of hatching eggs is a common practice in the poultry industry. This occurs because hatching eggs are not incubated immediately after oviposition for several reasons. Since eggs are not commercially incubated on farm they must be transported to commercial hatcheries. In Alberta, there are only three commercial hatcheries, Lilydale, Maple Leaf and Sunshine hatcheries. These hatcheries are located in the southern part of the province and are therefore far apart from the farms that produce the eggs. In addition, most of the farms are relatively small and as such produce small number of eggs per day. Eggs must therefore be stored on-farm since daily delivery of eggs to the hatchery is not practical. The eggs are also stored on-farm to cut down the cost of transportation. Eggs may also be stored at the hatchery for several reasons. When hatching eggs are plentiful, incubator space may also be limited; surplus eggs are stored until incubator space is available. Eggs may also be stored until bulk amounts are ready to fill large incubators to capacity. Storage in incubators operating at capacity will also save energy. Eggs are also stored if fewer chicks or poults are not required for placement on grow-out farms. During the hot summer months in the southern United States hatching egg production normally declines. In this case, hatchery managers anticipate a reduction in egg production due to high temperatures (Christensen et al., 2003). Small batches of eggs that arrive at the hatchery are

accumulated to maintain enough eggs for setting and subsequent chick or poult placement on grow-out farms.

During storage, hatching eggs are held at temperatures below which no appreciable embryonic development occurs. These temperatures are termed the physiological zero. Although at the physiological zero, embryonic development may be brought to a halt, metabolic activities continue. The exact temperature regarded as the physiological zero has been debated upon for many years. Edwards (1902) was the first to provide a temperature which appears acceptable to depict the physiological zero. Edwards (1902) noted that previous studies had placed the physiological zero between 25 and 34^{o}C (Dareste, 1891; Rauber, 1884). Dareste (1891) particularly placed the physiological zero at 28^{o}C and emphatically stated that every observation made showed that no blastodermal development took place below 28^{o}C. The author also stated that any embryo development that had already commenced was fatally arrested at a certain point between 30^{o}C and 34^{o}C.

However, Edwards (1902) reported that when eggs were placed between 20 and 21^{o}C, it was observed that at 20.05^{o}C and 20.72^{o}C normal development was 0.07% and 0.54%, respectively. To further prove that there was embryonic development at 21^{o}C, he incubated some eggs between 21 and 22^{o}C and noticed that at 21.08, 21.11, 21.38 and 21.87^{o}C normal embryonic development was 0.56, 1.07, 1.25, and 2.33%, respectively. Edwards (1902) then placed the physiological zero at 20^{o}C and assumed that below this temperature there will be no embryonic development. To verify that the physiological zero was actually below 20^{o}C, eggs

were placed at 17 to 18^{o}C for varying periods of 1 to 17 d (Edwards, 1902). Using the diameter of the blastoderm to determine if the embryo is growing, it was concluded that the given temperatures did not influence growth either during short or long periods and that the physiological zero was above this temperature (Edwards, 1902). Later on Funk and Biellier in 1944 established that the physiological zero lies between 25 and 27^{o}C or below 28^{o}C. The latest research to establish a physiological zero for the chicken development was by Fasenko et al. (1992). In this study, eggs were collected within 1 h of oviposition and assigned randomly to one of 5 storage treatment groups of 0, 4, 7, 14, or 21 d prior to incubation. Fresh egg weight was recorded and the eggs were placed on plastic egg flats and stored at 14^{o}C. Fasenko et al. (1992) realized that while the storage duration did not have any effect on embryonic development, storing eggs at this temperature (14^{o}C) stopped all embryonic development.

In current commercial incubation settings eggs are stored between 15^{o}C and 18^{o}C but it is acceptable that this temperature does not affect embryonic development upon incubation. Fasenko et al. (2001b) reported that it is important to store eggs at temperatures well below physiological zero to prevent abnormal growth of the embryo. Meijerhof (1992) reported that during egg storage, hatchability could be influenced by the length of the storage period, temperature, humidity, gaseous environment and the orientation of the eggs. Under higher storage temperatures, a decrease in hatchability or embryo viability can be detected even in eggs stored for 2 to 3 d or more (Bakst and Akkufo, 1999;

Fasenko et al., 2001a, b; Meijerhof, 1992). Therefore, storage temperature should be decreased with extended length of storage (Meijerhof, 1992).

3.6.1. Effects of egg storage on egg components

Scott and Silversides (2000) showed that as duration of egg storage increased in 31 wk old hens from 1, 3, 5, and 10 d, the percentage of yolk increased accordingly. This could possibly be due to albumen moisture loss. Although albumen weight and height also decreased as expected, the albumen pH increased with egg storage (Scott and Silversides, 2000). Eggshell weight did not follow any trend as storage time increased. Both albumen height and albumen pH can be used to determine albumen quality. Fasenko et al. (2009) reported that in eggs stored for 14 d versus. 1 d, the inner and outer perivitelline layers were very fragile in eggs stored for 14 d. The authors also observed that the thicknesses of the inner perivitelline layer and the dense area of the outer perivitelline layer were reduced in eggs stored for 14 d compared to eggs stored for 1 d. Other studies have also reported that the density of the inner and outer perivitelline dense layers decreased in eggs stored for 14 d versus 1 d stored eggs (Fasenko, 1996). Fasenko et al. (2009) also observed differences in the color of the yolk of long term stored eggs (14 d) and short term (1 d) stored eggs. The authors explained that the possible mechanism for these changes could be due to water movement from the albumen into the yolk in long-term stored eggs (14 d) compared to short term (1 d) stored eggs, resulting in the weakening of the perivitelline complex (inner and outer layers). Since perivitelline complex is vital for the development of the

embryo any change in its structure and density will lead to poor early embryonic development. This could also be a reason for higher early embryonic deaths in long-term stored eggs (Fasenko et al., 2009).

3.6.2. Effects of egg storage on hatchability and embryo mortality

The hatching egg industry has long recognized that egg storage longer than 7 d is detrimental to hatchability (Walsh et al., 1995). Previous research has shown that embryos from broiler eggs stored for 14 d had reduced embryonic growth rates and poorer broiler chick quality (Fasenko et al., 2001b). Additional results showed that, hatchability of eggs stored for 4 d compared to 14 d dropped from approximately 90% to 71% while embryonic mortality increased from about 8% to 21 % (Fasenko et al., 2001b). The same negative effects of long-term storage have also been observed in turkeys (Fasenko et al., 2001a).

Holding temperature as well as storage time can significantly influence early and late embryo mortality. Scott and Mackenzie (1993) reported that for freshly laid broiler eggs held for 24 h at 30°C and stored for 7 d, embryo development at 0 d of incubation was highest compared to eggs held at 18^{o}C for 24 hr and stored for 7 d. Embryo staging, weight and length of embryos at 3, 6 and 9 d following incubation were positively correlated with the holding temperatures and storage durations. The study shows that any temperature above acceptable physiological zero (20^{o}C) appeared to indicate some amount of embryonic development. However, the hatchability of fertile eggs was lowest (66.5%) for eggs held for 24 h at 30°C and stored for 7 d compared to eggs held at

18^{o}C. Earlier, Whitehead et al. (1985) noted that in broiler eggs stored for 14 d prior to incubation the hatchability was depressed by 10%. The authors further noted that most of the increases in embryonic mortality (64%) occurred during the first wk of incubation, with a further 30% occurring during the third wk of incubation. In turkey eggs, Fasenko et al. (2001a) reported that the hatchability of all eggs was depressed by 5.2 % while, the hatchability of fertile eggs decreased by 6.4% after storing eggs at 14^{o}C for 4 d and 14 d prior to incubation. In addition first wk mortality (1 to 7 d) of incubation and mortality at internal and external pipping (26 and 27 d) were significantly higher in eggs stored for 14 d compared to 4 d stored eggs.

3.6.3. Effects of egg storage on embryo vitality

Storage of eggs prior to incubation leads to morphological changes in embryo (Bakst and Gupta, 1997; Bakst and Akuffo, 1999), slows growth rate (Singal and Kosin, 1969), leads to shrinkage of the blastoderms (Edwards, 1902), delays initiation of development (Mather and Laughlin, 1979) and brings about differential development of different embryonic tissues (Kaufman, 1948). Embryonic survival rates were similarly depressed following extended storage of eggs (Meijerhof, 1992). In chickens, a healthy embryo contains averagely 60,000 embryonic cells after oviposition (Etches et al., 1996; Petitte et al., 1990). But Foulkes (1990) observed that as egg storage duration increased to 2 wk blasotdermal cell numbers reduced (freshly laid eggs = 50,908 cells; 2 wk stored eggs = 42,508 cells). Similarly, Bakst and Akuffo (1999) showed that in turkeys

as egg storage duration increased, blastodermal cell numbers also decreased. Their research established that blastodermal cell numbers from freshly laid turkey eggs declined from 32,000 to 21,500, 19,000 and 21,000 cells after storage for 2, 4 and 14 d, respectively. In addition, embryos from eggs which had been stored longer than 5 d at 15^{o}C to 18^{o}C and at room temperature for more than 36 h often had an anomalous appearance (Bakst and Akuffo, 1999). Earlier, Fasenko et al. (1992) hypothesized that there may be a minimum number of healthy embryonic cells required to initiate normal embryonic development or maintain the embryo in a viable state, and that egg storage may have reduced healthy embryonic cell numbers below a critical threshold resulting in abnormal development or dead embryos. Beside blastoderm, Kosin and St. Pierre (1956) showed that storage of broad breasted bronze turkey hatching eggs from 8 d to 14 d resulted in a lowered mean somite count after 60 h of incubation, as compared with eggs held for 1 to 7 d. All these studies appear to suggest that the impact of egg storage on embryonic viability can be before and during incubation.

Bloom et al. (1998) reported that when chicken eggs were stored for 14 d at 12^{o}C, 14% of cell death was due to apoptosis (programmed cell death). In 1999, Bakst and Akuffo also agreed that apoptosis and necrosis (injurious cell death) could be the mechanisms leading to cell death during eggs storage. Between freshly laid eggs and eggs stored for 2 d, Bakst and Akuffo (1999) could not exactly provide the reason for a 30% loss of embryonic cells within 48 h after egg storage. While the negative effects of egg storage remains a big concern in poultry industry, the exact biological mechanisms leading to cell death and

lowered productivity still remains mystery (Bakst and Akuffo, 1999; Bakst, 2003).

3.7. Embryonic cell death

Life and death decisions are taken by individual cells based on their interpretation of physiological signals and internal damage due to internal factors (intrinsic) and extracellular or environmental signals (extrinsic). This knowledge that cell death could be a genetically regulated process is worth investigating in order to manipulate the cell's inherent potential to control diseases, improve the cell's environment for proper development and improve productivity (McKenna et al., 1998). While death is an inevitable fact of life for all living organisms and in many cases, an important and predestined fate of individual cells, in this thesis two forms of cell mechanisms, apoptosis and necrosis were investigated due to an ongoing speculation in poultry research that appear to suggests that the two cell death mechanisms may play economically important roles in embryonic cell viability and embryo quality even before and during incubation (Foulkes, 1990; Bloom et al., 1998; Bakst and Akuffo, 1999). The goal was therefore to figure out how these limit or promote cell death.

Apoptosis is a biological mechanism through which cells in a multicellular organism commit suicide without any deleterious effect on neighbouring cells. It is a form of cell death initiated by the cell itself, genetically regulated, and executed using elaborate mechanisms of cellular and molecular machinery (Arnoult et al., 2002). For this reason, the term apoptosis is often used

interchangeably with the term "programmed cell death" (PCD) although technically, apoptosis is but only one particular form of PCD's often called PCD type I.). The other forms of PCD are autophagy or PCD type II (the process by which cells recycle cytoplasm and dispose of defective organelles), and nonlysosomal cell disintegration also called PCD type III (Díaz et al., 2005). Some examples and benefits of apoptosis include the destruction of cells infected with viruses, DNA damage, cancerous cells, and cells of the immune system after they have fulfilled their functions. Other developments where apoptosis is useful include the re-absorption of the tadpole tail at the time of its metamorphosis into a frog, the degeneration of web cells during the formation of fetus fingers, the sloughing off of the inner lining of the uterus (the endometrium) at the start of menstruation, and the removal of surplus cells and formation of proper connections (synapses) between neurons in the brain (http://www.dentalindia.com/apoptosis.html). Necrosis however, is believed to be a more disorderly type of cell death compared to apoptosis (Kerr et al., 1972). In addition to all the PCD types and necrosis, necroptosis (characterized by necrotic cell death morphology and activation of autophagy) and endoplasmic reticulum (ER) stress-induced cell death have also been reported (Diaz et al., 2005).

3.7.1. Definition of apoptosis and necrosis

The term apoptosis was first introduced into the public domain by Kerr et al. (1972) to describe a specific morphological pattern of cell death. Kerr et al. (1972) also reported that the morphological features suggest that it is an active,

inherently programmed phenomenon, and can be initiated or inhibited by a variety of environmental stimuli, both physiological and pathological. The mechanism was observed in different situations such as during embryonic development, normal cell turnover in healthy adult tissue, and atrophy upon hormone withdrawal. Kerr et al. (1972) grouped the structural changes that take place during apoptosis into two discrete stages. The first stage comprises of nuclear and cytoplasmic condensation and breaking up of the cell into a number of membrane-bound, ultrastructurally well-preserved fragments. In the second stage these apoptotic bodies are taken up by other cells, where they undergo a series of changes resembling in vitro autolysis within phagosomes, and are rapidly degraded by lysosomal enzymes derived from the ingesting cells. The authors suggested that the deletion of cells with little tissue disruption coupled with the absence of inflammatory responses allows reutilization of cellular components. As a result the morphological characteristics of apoptosis were proposed to result from a general mechanism of controlled cell deletion, which plays a complementary role to mitosis and cytokinesis in maintaining stable cell populations within tissues (Kerr et al., 1972).

Necrosis however, is death of a cell or group of cells as a result of injury, disease, or other pathologic state. In cells undergoing necrosis, there is swelling of cells and organelles, the cellular membrane is damaged and there is a release of intracellular contents which results in inflammation. Krysko et al. (2006) reported that there is lack of signaling from dying cell when it is necrotic and therefore makes it harder for the immune system to locate and recycle dead cells (necrotic)

than if the cell had undergone apoptosis. In higher animals, necrotic death of cardiac myocytes is a major contributor to heart failure (Tavernarakis, 2007). While necrotic cell death was traditionally considered a passive and chaotic process, emerging evidence indicates that specific molecular mechanisms trigger cellular destruction during necrosis (Syntichaki and Tavernarakis, 2002; Tavernarakis, 2007). Although the author (Tavernarakis, 2007) acknowledged that serious consideration should be given to developing mechanisms to stop necrotic cell death, there was no clear cut contribution to how it should be controlled once it is initiated except some preventive measures. In another study, it has been shown that both apoptotic and necrotic cells can be recognized and phagocytosed by macrophages (Brouckaert et al., 2004). In both cases, phagocytosis occurred through a phosphatidylserine-dependent mechanism, suggesting that externalization of phosphatidylserine is a general trigger for clearance by macrophages. However, uptake of apoptotic cells was more efficient both quantitatively and kinetically than phagocytosis of necrotic cells (Brouckaert et al., 2004). Using electron microscopy the authors showed clearly the morphological differences in the mechanisms used by macrophages to engulf necrotic and apoptotic cells. Here it was discovered that whereas apoptotic cells were taken up as condensed membrane-bound particles of various sizes rather than as whole cells necrotic cells were internalized only as small cellular particles after loss of membrane integrity (Brouckaert et al., 2004). In addition, a model uptake of neither apoptotic nor necrotic cells by macrophages modulated the

expression of proinflammatory cytokines by the phagocytes suggesting that with the right mechanisms, necrotic cell death can be controlled.

3.7.2. Mechanisms of cell death by apoptosis and necrosis

Members of the caspase family of proteases transmit the events that lead to apoptosis of animal cells. Distinct members of the family are involved in both the initiation and execution phases of cell death, with the initiator caspases being recruited to multicomponent signaling complexes. Initiation of apoptotic events depends on the ability of the signaling complexes to generate an active cysteine protease (Salvesen and Dixit, 1999). These proteases are present as inactivated enzymes (or zymogens) in essentially all animal cells, but can be triggered to assume active states, generally involving proteolytic processing as conserved aspartic acid (Asp) residues. During activation, the zymogen pro-proteins are cleaved to generate the large and small subunits of the active enzymes with two active sites per molecule (Reed, 2000). It is well established that certain caspases (caspase-8, caspase-9, and caspase-10) play upstream ‘‘initiator’’ roles in apoptosis by coupling cell death stimuli to the downstream ‘‘effector’’ caspases (caspase-3, caspase-6, and caspase-7).

The initiator caspases are highly specific proteases that cleave few proteins other than their own precursors and the downstream effector caspases (Slee et al., 1999). Thus, the bulk of the proteolysis that takes place during apoptosis appears to be carried out by the effector caspases (Rodriguez and Lazebnik, 1999). However, the relative contributions made by the effector

caspases to the demolition phase of apoptosis are still largely unknown (Walsh et al., 2008). Though many pathways for activating initiator and executioner caspases may exist (KEGG, 1995-2010), only two have been elucidated in detail. One of these centers on tumor necrosis factor (TNF) family receptors, which use caspase activation as a signaling mechanism, and connecting ligand binding at the cell surface to induce apoptosis (Yuan, 1997). Another pathway involves the participation of mitochondria, which release caspase-activating proteins (or cytochrome c) into the cytosol, thereby triggering apoptosis (Reed, 1997).

The death receptor and mitochondrial pathways for caspase activation are sometimes referred to as extrinsic and intrinsic apoptosis pathways respectively. In the events leading to the activation of death effector caspases during apoptosis, Walsh et al. (2008) provided an oversimplification schematic representation of the process that occur during the intrinsic and extrinsic pathways in vivo. The extrinsic pathway can be induced by members of the TNF family of cytokine receptors, such as TNFR1 (tumor necrosis factor receptor 1) and Fas (apoptosis stimulating fragment). These proteins recruit adapter proteins to their cytosolic death domains proteins (DD), including Fadd (Fas-associated death domain), which then binds death effector domain proteins (DED), containing pro-caspases, particularly pro-caspase-8. According to Walsh et al. (2008) the intrinsic pathway involves the release of cytochrome c from mitochondria and activation of various stimuli, including elevations in the levels of pore-forming pro-apoptotic *Bcl-2* (B-cell lymphoma) family proteins such as *Bax (*Bcl-2-associated X protein), *Bad* (Bcl-2 associated death promoter protein), *Bak* (Bcl-2 homologous

antagonist/killer protein), *Bok* (Bcl-2 ovarian killer protein) and *Bid* (BH3 interacting domain death agonist protein). In the cytosol, cytochrome c binds and activates apoptotic protease activating factor 1 (Apaf-1). Apaf-1 then binds to pro-caspase-9 leading to it activation. Activation of caspase-9 (intrinsic) and caspase-8 and -10 (extrinsic) have been shown to directly cleave and activate the effector proteases, caspase-3. Other caspases such as caspase-6 and -7 can also become involved in these pathways as downstream effector caspases. However, depletion of either caspases in some experiments had minimal impact on any of the parameters investigated, calling into question their precise role during the execution phase of apoptosis (Slee et al., 2001).

Matsumura et al. (2000) showed that the death effector domain of Fadd was responsible for the Fadd-mediated necrotic pathway. Though they reported that the process was accompanied by a loss of mitochondrial transmembrane potential, there was no release of cytochrome c from the mitochondrion. The molecular mechanism of necrosis and its regulations appear to be recently understood (Brouckaert et al., 2004). However, previously suggested mechanisms include the release of lysosomal enzymes, the generation of toxic O_2 radicals, and the activation of calcium-dependent phospholipases (Fiers et al., 1999). The morphological features of a cell death either by apoptosis or by necrosis are remarkably conserved for quite different cell types derived from lower or higher organisms. At the molecular level, several gene products have been shown to play similar, but crucial roles in the major cell death pathways in both worm and human (Fiers et al., 1999). It is evident that there are multiple pathways leading to

cell death (KEGG, 1995-2010). However, some cells may have the required components for one particular pathway. This is because some cells may contain endogenous inhibitors which preclude a particular pathway. Furthermore, different pathways can co-exist in the same cell and are switched on by specific stimuli (Fiers et al., 1999; KEGG, 1995-2010).

3.7.2. Methods of assessing cell death by apoptosis and necrosis

There are different ways to measure cell death. These include typical qualitative methods such as DNA fragmentation, trypan blue exclusion test and microscopy (Loo and Rillema, 1998). Other traditional cell-based methods include light and electron microscopy and nuclear stains. However, flow cytometry methods are the most popular in assessing cell death due to their reliability. Even complex flow cytometers which also assess cellular morphology in addition to providing quantitative information have become available. Compared to the classic methods of DNA ladder formation by gel electrophoresis and of morphologic examination by electron microscopy for determination of apoptosis, flow cytometry permits rapid and quantitative measurements on apoptotic cells. Many different flow cytometric methods for the assessment of apoptosis in cells have been described (Darzynkiewicz et al., 1992). Most of these methods measure apoptotic changes in cells after uptake of various cellular membrane or DNA dyes. Other techniques using the terminal deoxynucleotidyl transferase or nick translation assays have also been developed which when used in conjunction with standard flow cytometric staining methods may yield

informative data relating cell death to various cellular parameters, including cell cycle and cell phenotype Gorczyca et al., 1993). The use of one or more of the methods described in this chapter for measuring cell death should enable investigators to accurately assess apoptosis in the context of the various models being examined and help define causal relationships between the mechanisms that regulate apoptosis and the cell death event itself (Loo and Rillema, 1998).

It has been postulated that the stability and the kinetics of intermediate cell death stages may determine the resistance of a particular cell type to DNA degradation. Consequently, the visibility of "sub-G1" peaks (indicative of apoptosis) (Wang et al., 2003) by flow cytometry analysis and also the formation of characteristic DNA ladders on agarose gels though might be a function of individual cellular homeostasis or ensure cell survival, the absence of a "sub-G1" peak on a DNA histogram should not constitute proof of no apoptosis (Ormerod et al., 1992). On the contrary, the mere appearance of a hypodiploid DNA peak should not also be taken as definite evidence of the presence of apoptotic cell population without other supporting information (Ormerod et a., 1992). Flow cytometric findings in a particular cell type exposed to certain stimulus must therefore be verified always with other non-flow cytometric methods. Lately, rapid flow cytometric staining methods that use annexin V-fluorescein isothiocyanate (annexin V) for detection of phosphatidylserine exposure on the cell surface as a marker of apoptosis have become commercially available. For this staining method it is essential to add a dead cell discrimination dye like propidium iodide or 7-amino-actinomycin D to the stained cells. Because late

apoptotic or necrotic cells also express phosphatidylserine and they have to be distinguished from the early apoptotic cells by different fluorescence (Vermes et al., 1995). The newest flow cytometric assays measure Caspase-3 activity, an early marker of cells undergoing apoptosis and kits for performing these assays are commercially available (Nicholson et al., 1995).

3.8. Molecular mechanisms of embryonic survival

The information for the development of a complex adult organism from a relatively simple fertilized egg is contained within the deoxyribonucleic acid (DNA or genes). The genes are responsible for protein biosynthesis. This is accomplished through the interplay of the ribonucleic acids (RNA) and the DNA. In simple terms, protein biosynthesis begins with information copied from DNA onto the RNA (transcription). The information is then moved by the RNA from the nucleus into the cytoplasm and used to synthesize protein linear amino acid sequence or primary proteins (translation) (Massaro, 1969). However, various environmental factors are indispensable in the expression of these genes and their proteins for the embryo to achieve its full genetic potential. The expression of some of these genes coordinates the process of cell death while other genes promote the survival of the cell.

The survival of each cell depends on the equilibration of cell division (cell proliferation and differentiation) and cell death in order to maintaining proper cellular homeostasis (Ma et al., 2001). For example, the removal of cells by apoptosis must often involve the interplay of many genes classified as pro-

apoptotic (e.g. *Bax Bad, Bak* and *Bok*) and anti-apoptotic (e.g. *Bcl-2, Bcl-xL* and *Bcl-w*). While the anti-apoptotic genes act to protect the cell from dying, the pro-apoptotic genes lead to cell death, precisely apoptosis.

In mammals, Fujita (1999) noticed that cold shock response has attracted some attention in adaptive thermogenesis, cold tolerance, storage of cells and organs, and treatment of brain damage and protein production. Though at the cellular level, some responses of mammalian cells are similar to microorganisms, cold stress is reported to change the lipid composition of cellular membranes, and suppress the rate of protein synthesis and cell proliferation. However, this knowledge needs to be applied in the poultry industry to study the incidence of cold storage of hatching eggs at the cellular and molecular levels to improve embryo development. And biologically such a study is needed to explain the mechanisms behind reduced embryonic development, metabolism and cell death following cold storage of eggs prior to incubation.

3.9. A look at some key apoptosis genes

B-Cell Lymphoma (*Bcl-2*) gene and its anti-apoptotic partners like *Bcl-xL*, normally function as protectors against mitochondrial initiation of caspases. These genes encode an integral outer mitochondrial membrane protein that block the apoptotic death of some cells such as lymphocytes. The gene *Bcl-2* is known to suppress apoptosis in a variety of cell systems including factor-dependent lymphohematopoietic and neural cells. It regulates cell death by controlling the mitochondrial membrane permeability. It functions in a feedback loop system

with caspases, the proteins that executes apoptosis. Its main function is to inhibit caspase activity either by preventing the release of cytochrome c from the mitochondria and or by binding to the apoptosis-activating factor 1 (APAF-1) and preventing it from recruiting cytochrome c. Korsmeyer (1999) reported that transgenic mice that overexpressed *Bcl-2* demonstrated extended cell survival. The *Bcl-2*-deficient mice however, demonstrate full amount apoptosis of lymphocytes, profound renal cell death and loss of melanocytes.

The Bcl-2 protein duels with its counteracting twin, a partner known as *Bax*, a pro-apoptotic gene. When *Bax* was in excess, cells executed a death command; but, when *Bcl-2* gene dominates, the program is inhibited and cells survive. Bax-deficient mice display cellular hyperplasia, confirming its role as a pro-apoptotic molecule. An expanded family of *Bcl-2*-related proteins shares homology clustered within four conserved regions termed *Bcl-2* homology 1 through 4 (BH1-4). These novel domains control the ability of these proteins to dimerize and function. An amphipathic alpha helix, BH3, is of particular importance for the pro-apoptotic family members. *Bid* and *Bad* also represent an evolving set of pro-apoptotic molecules, which bear sequence homology to *Bax* but only at BH3. They appear to reside more proximal in the apoptosis pathway serving as death ligands. *Bad* connects upstream signal transduction paths with the *Bcl-2* family, modulating this checkpoint for apoptosis. In the presence of survival factor interleukin-3, cells phosphorylate *Bad* on two serine residues. This inactivated *Bad* is held by a different protein, freeing *Bcl-xL* and *Bcl-2* to promote survival. Activation of *Bax* results in the initiation of apoptosis. Downstream

events in this program include mitochondrial dysfunction, as well as Caspase activation.

The pro- and anti-apoptotic *Bcl-2* family members represent central regulators in an evolutionarily conserved pathway of cell death. Aberrations in the *Bcl-2* family result in disordered homeostasis, a pathogenic event in diseases, including cancer. In a related study, the expression of *Bcl-xL* gene normally implicated with *Bcl-2* gene in cell death was studied by Northern and Western blot. When apoptosis was correlated with *Bcl-xL* mRNA and protein down-regulation, it was observed that overexpression of *Bcl-xL* reduced the amount of apoptosis by 80% when compared with control. In addition, the overexpression of *Bcl-xL* prevented the activation of caspase-9 and caspase-3. The results concluded that down-regulation of *Bcl-xL* expression mediates apoptosis (Blanco-Colio et al., 2003). The anti-apoptotic effect of *Bcl-xL* also contributes to the inhibition of a direct cytotoxic effect of pro-apoptotic family members such as *Bax* and *Bak* (Oltvai et al., 1993). Down-regulation of the anti-apoptotic genes satisfies the action of the pro-apoptotic genes that promote activation of caspases (proteases). These caspases contribute to apoptosis through disassembly of a cell structure by cleaving several proteins involved in cytoskeleton regulation and destruction of nuclear lamina (Thornberry et al., 1998). The pro-apoptotic genes, particularly, *Bax* and *Bak* have been shown to function at the endoplasmic reticulum membrane to activate transmembrane protein kinase and endoribonuclease inositol-requiring enzyme-1alpha (IRE1alpha) signaling and to provide a physical link between members of the core apoptotic pathway (Hetz et al., 2006).

Bok is also one of the pro-apoptotic *Bcl-2* family proteins (genes) with restricted and heterodimerization properties that facilitate elucidation of apoptosis mechanisms in tissues undergoing hormone-regulated cyclic cell turnover. In the intracellular death program, hetero- and homodimerization of different anti- and pro-apoptotic *Bcl-2*-related proteins are critical in the determination of cell fate. From a rat ovarian fusion cDNA library, Hsu et al. (1997) isolated Bok as a relatively new pro-apoptotic *Bcl-2* gene. It was named *Bcl-2*-related ovarian killer (*Bok*). They observed that *Bok* has conserved *Bcl-2* homology (BH) domains 1, 2, and 3 and a C-terminal transmembrane region present in other *Bcl-2* proteins, but lacked the BH4 domain found only in anti-apoptotic Bcl-2 proteins. In mammalian cells, overexpression of *Bok* was shown to induce apoptosis that was blocked by the baculoviral-derived cysteine protease inhibitor *p35*. In situ hybridization analysis of *Bok* showed that its mRNA was localized in granulosa cells, the cell type that underwent apoptosis during follicle atresia.

In humans, the interplay between *Bcl-2* and *Bax* proteins is a key process in determining if apoptosis takes place. The two proteins have similarities in structure, so *Bax* can prevent expression of *Bcl-2* by binding with it. *Bcl-2* is found in the mitochondria, a cellular structure important for energy formation. The *Bcl-2* protein inhibits apoptosis by preventing the release of cytochrome c protein from the mitochondria into the cytosol. When irreversible damage occurs to the cell, the protein 53 (or *p53*) gene stimulates the formation of *Bax* protein, which promotes apoptosis by releasing cytochrome c. This process results in the activation of caspase enzymes that kill the cell by breaking down its constituent

proteins. In B-cell leukemia and lymphoma, there is a common chromosomal abnormality. The *Bcl-2* gene region on chromosome 18 is moved to the immunoglobulin heavy chain region on chromosome 14. Although normally responsible for the production of large amounts of antibodies, the fusion gene now produces excessive *Bcl-2* protein. This over expressed *Bcl-2* prevents apoptosis; so damaged cells survive and could progress to a cancerous growth (KEGG, 1995-2010).

3.10. Concluding remarks

From the literature review, there is convincing evidence that the development of the avian embryo particularly chicken and turkey are influenced by many factors. Some of these factors do not act alone but through a multifaceted chain of events that determine the fate and survival of the embryo. Christensen et al. (2001b) discovered that among the three factors that are being investigated in this thesis (genetic strain, maternal flock age and egg storage), the rate at which glycogen was accrued into muscle and heart tissue displayed a significant three-way interaction among genetic strain, maternal flock age, and egg storage. Particularly, embryos from the line that resisted storage mortality maintained greater glycogen concentrations in muscle and heart tissues than those from the genetic line and the flock age with diminished survival rates. It was concluded that embryonic survival rates differ following egg storage because of the ability of some embryo to accrue and maintain adequate carbohydrate for growth and function of vital demand tissues. Because genetic selection and

breeding experiments have been intensively used to increase the productivity of current and modern broiler and turkey genetic strains, the effects have become very significant within other factors such as flock age and egg storage that have not been considered during those breeding programs. However, with the realization that more is expected from these birds at the expense of their healthy growth, poultry scientists in the current era need to go back and reconsider cellular and molecular strategists that can be used to reduce if not to reverse the adverse effects already planted by continuous selection in the past 40-50 years. Since numerous embryo parameters "growth rate, embryo weight, hatch time, embryo mortality, absence or presence of abnormalities, and metabolic and respiratory rates on whole embryo as well as blastoderm morphology, blastodermal cell death and survival, and gene expression" that combine to determine embryo quality may be influence by a lot of factors before and during incubation, the persistent question embryologists need to address is: by what fundermental physiological, cellular and molecular mechanisms do these factors that limit embryo quality determine the fate of the embryo during egg storage and incubation. In the current thesis three of these key factors (genetic strain, maternal flock age and egg storage) that typically limit smooth embryonic development and embryo quality during commercial incubation were investigated because of the industrial potential that exist to used them to increase farm and hatchery performance in the broiler and turkey production chain.

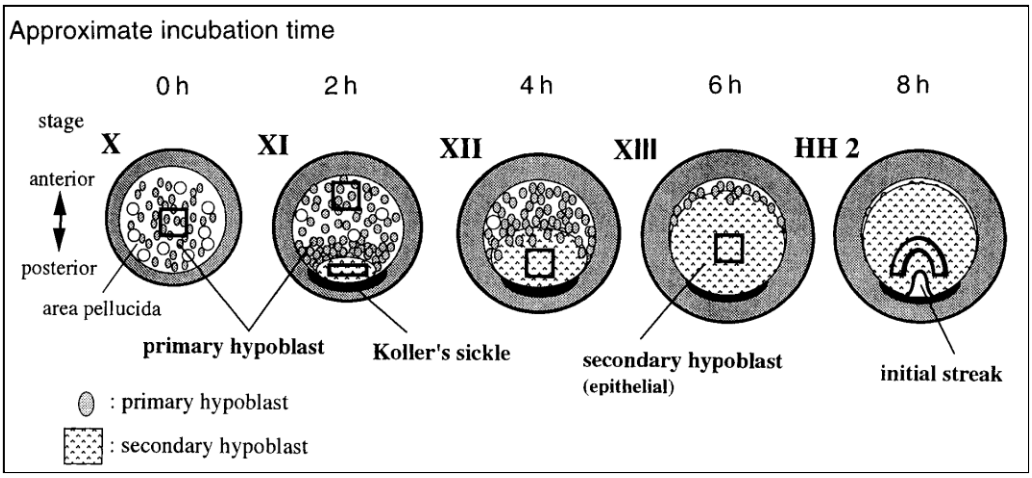

Figure 1.1. Blastoderm development after oviposition. The illustrations show the regions of chicken blastoderm from Eyal-Giladi and Kockav (1976) stage X at various stage of embryonic development during the first 8 h of incubation. Source: Mogi et al. (1998).

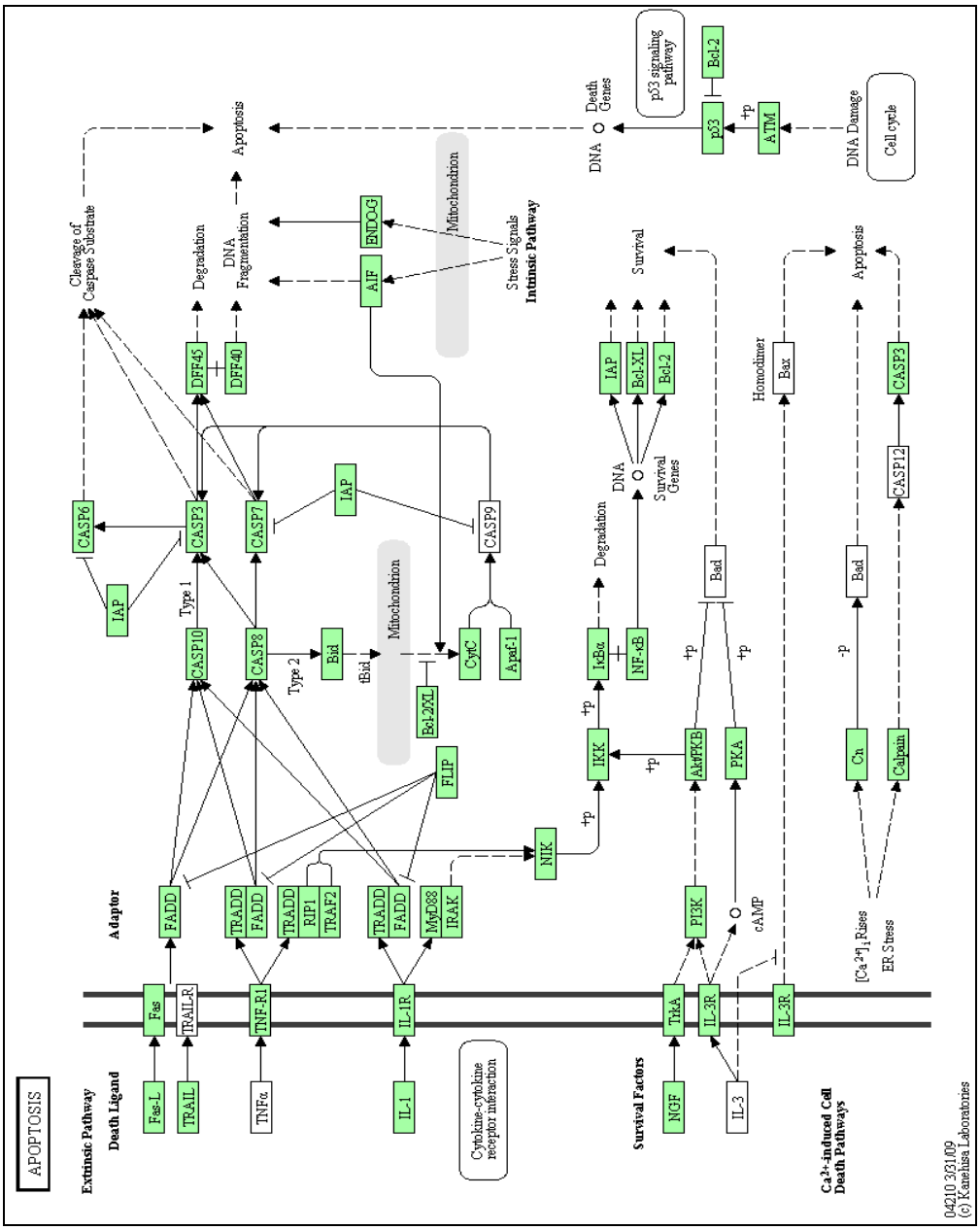

Figure 1.2. Kyoto Encyclopedia of Genes and Genomes (KEGG, 1995-2010) pathways of apoptosis in the domestic fowl (Gallus gallus). In this figure there are about 5 pathways through which apoptosis can occur in the chicken cell. These include extrinsic and intrinsic pathways that use death ligand and mitochondrial materials to initiate apoptosis. Other pathways are the cytokine-cytokine receptor interaction, calcium induced cell death pathways and the p53 signaling pathway.

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CHATPER 2: GENERAL METHODS AND MATERIALS

1. INDIRECT CALORIMETRY SYSTEM

1.1. Detailed description of the embryonic metabolic system

The metabolic system used in this thesis is a modification from one previously used by O'Dea et al. (2004) and Segura et al. (2006) with the incorporation of a differential O_2 analyzer (DOX) (Qubit Systems Inc., Kingston, ON). The operation and analysis of experimental data using this calorimetry system in broiler embryonic metabolism has also been established in a previous study (Hamidu et al., 2007). In detail, the indirect calorimetry system consists of 24 metabolic chambers each connected to a three-way electric activated solenoid valve (ASCO Valve Canada, Brantford, ON), that is located outside an incubator. The valves control air flow through the metabolic chambers (Figure 2.1). These valves were operated by a computer driven relay switcher located in a valve control box housed on top of the incubator. When a valve was switched on, the air flow from that particular metabolic metabolic chamber was directed into two different vacuum manifolds for analysis, first; the CO_2/H_2O analyzer (Model LI-6262, LI-COR Inc., Lincoln, NE) and then the DOX. The air sampled from each chamber was drawn into the CO_2/H_2O analyzer every 5 min using a sample pump (Qubit Systems Inc. 4000 Bath Road, 2nd Floor, Kingston, ON) set daily at a constant flow rate. When a valve was switched off the air was directed to the exhaust port. In this way the air flow through one metabolic chamber could be monitored, while air from the other 23 metabolic chambers were exhausted

outside from their chambers. The O_2 and CO_2 readings were automatically averaged during the last 10 s of the 5 min period for each metabolic chamber.

Airflow through the metabolic chambers was adjusted daily by flow meters in line with the solenoid valves. These flow meters were calibrated at the beginning of each experiment by a standardized external flow meter (Dry Cal) (Qubit Systems Inc. 4000 Bath Road, 2nd Floor, Kingston, ON). While the CO_2/H_2O analyzer operated from absolute mode in recording CO_2 data, the DOX was set up in a differential mode such that O_2 concentration was based on electronic difference between O_2 partial pressures between the sample $(PO_2)_S$, kPa) and the reference $(PO_2)_R$, kPa) sensors and the prevailing atmospheric pressure (Atm, kPa), as shown in the equation (1) below. The differential O_2 pressure (kPa) was expressed as parts per million (ppm). The O_2 and CO_2 exchange rates were calculated as demonstrated (Equation 2)

$$\text{Diff } O_2 \text{ (ppm)} = \frac{(PO_2)_S - (PO_2)_R}{Atm} \times 10^6 \quad [1]$$

$$\text{Gas exchange rate (ml/d)} = \frac{O_2 \text{ or } CO_2 \text{ concentration (ppm) x flow rate (ml/d)}}{10^6} \quad [2]$$

Only 25ml/min of air was drawn from the CO_2/H_2O analyzer (sample air) or the incubator (reference) entered into the DOX. Air through the DOX was operated by two DOX controller pumps (Qubit Systems Inc. 4000 Bath Road, 2nd Floor, Kingston, ON). One pump controls metabolic air coming from the CO_2/H_2O analyzer to the DOX sample air port. The other pump withdrew a sample of air coming directly from the incubator into the reference port of the DOX. Both pumps were set to draw only 25 ml/min of air through each DOX

inlets port. In this way a zero differential pressure between the sample and reference cells of the DOX can be attained to prevent damage to the DOX active cells.

The entire metabolism set up, where changes in CO_2 and O_2 concentrations due to respiration are monitored continuously and not in separate pathways, is called the open flow mode. Daily calibration of the CO_2/H_2O analyzer and the DOX were done with certified nitrogen gas and certified span gases with the following composition (CO_2 = 3038 pmm, O_2 = 21.02 %) and balanced with nitrogen. The detailed daily calibration of the calorimetry system has been shown in appendix 1, while the validity of the calorimetry system, O_2 and CO_2 analyzer, and the flow rates have been established (Hamidu et al., 2010).

1.2. Verifying the accuracy of the metabolism system

To ensure that the CO_2 analyzer and the differential O_2 analyzer were providing accurate measurements, butane, which, has a theoretical RQ value of 0.62 (Nunn et al., 1989) was used as a source of combustion to simulate metabolic respiration of an avian embryo (Hamidu et al., 2010). For this test two of the 24 metabolic chambers were used to test the reliability of the CO_2 analyzer and the DOX (Figure 2.2). The air sampled from chamber 1 was drawn from an available air source and used as the reference air. The sample air from chamber 2 was drawn from a butane combustion chamber located outside the incubator. The air for combustion was provided by the same air source as the reference and was used to maintain the butane flame burning. Both the reference air from chamber 1 and

butane sampling air from chamber 2 were drawn at flow rates ranging from 75 to 300 ml/min that were typically used for an avian embryo over a 21-d incubation period.

For the actual test a butane torch was used to burn gas at a level that produced a good analyzer response at airflow rates of 75, 100 and 300 ml/min through the metabolic chambers. The combustion air was drawn from the combustion chamber into metabolic chamber 2. As shown in Figure 2.2, the sample air from metabolic chamber 1 was considered to be the background concentrations prior to combustion. The chamber flow rates were used to calculate the O_2 consumption and the CO_2 production rates (ml/d) based on the O_2 and CO_2 differential concentrations (Equation 2). The accuracy of the flow rates calibrated on the flow meters was ± 5 ml/min. The RQ was calculated as the ratio of the CO_2 production rate to O_2 consumption rate (Krogh and Lindhard, 1920). These values were computed and compared to the theoretical RQ value of butane (RQ = 0.62).

2. EMBRYONIC METABOLISM

2.1. Effects of turkey genetic strain and flock age on embryonic metabolism

In order to use the calorimetry system to further investigate embryonic metabolism, freshly laid turkey hatching eggs were obtained from 4 commercial turkey farms via a commercial hatchery in Alberta. Eggs from 2 modern turkey genetic strains Hybrid (H) and Nicholas (N) were obtained at 4 parent flock ages:

Young (Y – 30 week), Peak (P – 34 week) Mature (M – 55 week) and Old (O – 60 week). The H eggs for Y and P flocks were obtained from the same flock (farm A) while, the H eggs for M and O flocks were obtained from another flock (farm B). Similarly, the N eggs from Y and P flocks were obtained from a single flock (farm C) while, N eggs for M and O flocks were obtained from another flock (farm D). All experimental procedures involving live embryos older than 6 d of incubation were approved by the Faculty of Agriculture, Forestry and Home Economics (currently, Faculty of Agricultural, Life and Environmental Sciences) Animal Policy and Welfare Committee at the University of Alberta, Edmonton, Alberta, according to the principles and guidelines outlined by the Canadian Council on Animal Care (1993).

2.1.1. Eggshell conductance

At each flock age, 15 hatching eggs per genetic strain were weighed and placed vertically with broad end up in 300 ml plastic containers as described by O'Dea et al. (2004). The eggs were completely covered with desiccant (Drierite, W. A. Hammond Drierite Company Ltd., Xenia, OH) and placed in a desiccator. A thermometer was placed inside the desiccator to record the daily ambient temperature. The average daily moisture loss from the eggs and the temperature of the desiccators were recorded daily for 9 d. The data recorded during the first 2 d were not used as it was regarded as an equilibrium period. The average temperature for 7 d was expressed as saturated vapor pressure and used to calculate the G value as described Ar et al. (1974) and illustrated below.

$$G_{H_2O} = \frac{M_{H_2O}}{\Delta P_{H_2O}} \quad [3]$$

Where, G_{H_2O} = water vapor conductance or eggshell conductance (mg/day/mmHg), M_{H_2O} = the rate of water loss from egg (mg/day), ΔP_{H_2O} = $P_{H_2O} - P_o$, P_{H_2O} = water vapor pressure in the egg, P_O = water pressure outside the shell (desiccant = 0), ΔP_{H_2O} = water vapor pressure difference across the shell which is equal to the saturated vapor pressure (mmHg).

At the 9th d all eggs were weighed, broken open, and the yolk and the eggshell separated from the albumen. After taking wet yolk and wet eggshell weights, albumen weight was calculated by subtracting wet yolk and wet eggshell weights from the final egg weight. The shell thickness was measured at the equator with a micrometer screw gauge (SR44 battery model, Mitutoyo, Aurora, IL). The egg components except the albumen were placed in a drying oven (Desptach V Series model VRC2- 26-1E, Despatch, Minneapolis, MN) at 65°C for 4 d to determine dry matter (DM) weight. All the egg components (wet and dry) were expressed as a percentage of initial egg weight.

2.1.2. Measurement of embryonic metabolism

At each flock age, 11 eggs per genetic strain were individually placed in one of 24 identical one 1L airtight metabolic chambers inside a James way AVN incubator (James way Incubator Company Inc., Cambridge, ON). Eggs were incubated at 37.5 °C and 56 % RH for 25 d. From 1 to 25 d of incubation the eggs

were held in place by a piece of plastic incubator egg flat cut to fit the bottom of each metabolic chamber. In each trial, 5 spare eggs per strain were placed in a separate incubator under the same temperature and RH conditions. After 7 d of incubation, the daily increase in O_2 consumption and CO_2 production of all the eggs in the metabolic chambers were assessed to determine whether all the eggs contained viable embryos. The eggs in metabolic chambers with daily increase in gas exchange were regarded as fertile and those with little or no consumption of O_2 and production of CO_2 were regarded as containing non-viable embryos (infertile or dead). These eggs were removed and replaced with spare eggs of the same genetic strain. At 25 d of incubation, the egg flats were removed from the metabolic chambers leaving each egg on its side for the last 3 d of the incubation. To simulate the conditions of a hatcher, the incubator turning mechanism was stopped; the temperature of the incubator was dropped to 36.94 ^{o}C and the RH was raised to 60% for the last 3 d of incubation. Beginning at 26 d of incubation, the metabolic chambers were checked every 6 h for signs of external pipping and hatched poults. All hatched and dry poults were removed and weighed; poult length (from the tip of the beak to the end of the longest toe) and shank length (metatarsus, measured from top of hock to foot pad) were measured. Each poult was euthanized by cervical dislocation and the residual yolk sac (RYS) removed. The wet yolk free body mass (YFBM) and RYS were weighed and placed in a drying oven (Desptach V Series model VRC2- 26-1E, Dispatch, Minneapolis, MN) at 65 °C for 4 d to determine the DM weight. After 28 d of incubation, all unhatched eggs were broken open to determine the approximate day of embryonic

death. The wet and dry YFBM and RYS weights were expressed as a percentage of poult weight at hatching.

Additionally, 270 eggs per genetic strain per flock age were weighed and incubated in a 5,000 egg capacity single-stage James way incubator (James way Incubator Company Inc., Cambridge, ON) under the same temperature and RH conditions as described previously above. From 4 to 28 d of incubation, the number of live embryos obtained from 15 eggs per strain per flock age broken open, was used to determine the average embryo weight for each day of incubation.

For each trial, embryonic O_2 consumption and CO_2 production were recorded from 22 of 24 metabolic chambers. For each day of incubation 6 repeated measurements of embryonic O_2 and CO_2 concentrations were recorded from 4 d of incubation to 28 d of incubation. During the first 7 d of incubation the flow rate was set at 100 ml/min. For the rest of incubation the air flow rate was set at 300ml/min. The embryonic heat production (EHP) was calculated using the formula shown provided by Kleiber, 1987 (Equation 3). The daily EHP from 4 d to 28 d of incubation was also expressed as embryonic EHP per gram embryo wet weight (EHP/g) by dividing daily EHP by the average daily wet embryo weight.

Embryonic Heat production (mW) = [3.871 x O_2 consumption (L/d) + 1.194 x CO_2 production (L/d)] x 1 d / 24h x 1 h / 3600s x 1000cal / 1 kcal x 4.187 J / cal x 1000 mW/W. [4]

2.2. Effect of broiler egg storage on embryonic metabolism

In a related study, 562 Ross 308 broiler breeder-hatching eggs were obtained from 33 week old flocks from a commercial hatchery. One group of 281 eggs were collected, weighed and stored for 14 d at temperature of 16-18^{o}C and 70-80 % RH. After 10 d, a second group of 281 eggs was collected, weighed and stored for 4 d under the same temperature and RH. The egg collection and storage practice were repeated when flock age reached 37 week. Similar to the above study (section 2.1.2), 11 eggs per storage treatment (4 d and 14 d) were used to monitor embryonic O_2 consumption and CO_2 production and used to calculate embryonic heat production. The procedures and equipment used for this experiment have been previously reported above. However, because chicken embryos take 21 d to hatch the plastic incubator egg flat that holds the egg in position was removed at 18 d of incubation to allow the eggs to hatch. In addition, each metabolic chamber was checked every 6 h from 19 d of incubation, and the approximate time of external pipping (signs of embryo breaking through egg) and hatching time recorded. Hatched chicks were removed from the chambers; each hatch chick was weighed and euthanized through cervical dislocation.

Likewise, a total of 270 eggs per storage treatment were incubated in separate incubator and transferred into a hatcher at 18 d of incubation. Beginning from 4 d up to 21 d of incubation, 30 eggs (15 eggs per storage treatment) were removed and opened to determine daily embryonic wet weight. The daily wet samples were dried in oven (Despatch V Series model VRC2-26-IE) at 65ºC for 4 d to obtain daily dry embryo weights. Embryonic O_2 and CO_2 concentrations were

expressed as O_2 and CO_2 exchange rates (Hamidu et al., 2007) and used to calculate embryonic heat production as shown above (Equation 3).

3. CELLULAR ANALYSIS

3.1. Develop techniques to separate blastoderms into single cells

3.1.1. Blastoderm preparation

In the first phase of the study, hatching eggs were obtained from white leghorn hens (Lohmann LSL-Lite strains ≥ 40 wk older) at the Poultry Research Center, University of Alberta and stored at 16^{o}C and 80% RH for 4 d. A total of 35-40 blastoderms were harvested individually into phosphate buffered saline without calcium and magnesium solution ($PBS^{-/-)}$ and dissociated into individual cells in trypsin/EDTA solution. Dissociated cells were stained with annexin V-fluorescein isothiocyanate (annexin V) and propidium iodide (PI) (Molecular Probes, Inc. 29851 Willow Creek Road, Eugene, OR). Apoptotic and necrotic cells were differentiated from live or viable cells using flow cytometer (BD FACScan, Becton Dickinson, San Jose, CA). Annexin V is a Ca^{2+} dependent phospholipid-binding protein that has a high affinity for exposed phospholipid-like phosphatidylserine (PS) (Vermes et al., 1995). The PS is usually kept on the inner-leaflet of the cell membrane by the enzyme translocase but when it is exposed to the outer layer, apoptosis inducing agents (normally high Ca^{2}+ conjugated molecules) binds permanently to PS, an indication of apoptosis events (Vermes et al., 1995). Propidium iodide however, can only bind the cell by intercalating between the double stranded DNA after the cell membrane becomes

permeable. Data obtained from flow cytometer procedures were reassessed by ImageStream multispectral flow cytometer system (Amnis Corporation, 2505 Third Avenue, Suite 210, Seattle, WA).

To obtain blastoderms for flow cytometry analysis, each egg was broken and the yolk separated from the albumen using standard egg separator (Fasenko et al., 1991). Excess albumen on the yolk was wiped off the blastoderm with kimwipe tissue paper (SPI Supplies/Structure Probe, Inc, 569 East Gay Street, West Chester, PA). The blastoderm was carefully covered with a filter paper ring (Petitte et al., 1990). The yolk membrane was cut around the filter paper ring and the blastoderm slowly lifted of the surface of the yolk at approximately a 45^{o} to 60^{o} angle. The blastoderm was gently rinsed in $PBS^{-/-}$ in wash glass to remove excess yolk. The blastoderm was transferred to a clean $PBS^{-/-}$ solution and cleaned under a microscope focusing between 20 and 60 x magnifications (Wild/Leitz-Wetzlar, Heerbrugg, Ireland). Excess yolk particles were dispersed by drop-wise release of $PBS^{-/-}$ solution from the surface of the wash glass onto the blastoderms. Blastoderms were further blown gently against the wash glass to further disperse small yolk particles. After cleaning, blastoderms with clearly defined areas pellucida and area opaca (Figure 2.3a) and classified as stage X (Eyal-Giladi and Kochav, 1976) were saved for further analysis. Each blastoderm was taken with an aliquot of $PBS^{-/-}$ into a 1.5 ml microcentrifuge tube (1.5 ml tube) kept at $4^{o}C$. The cleanliness of blastoderms isolated in the current thesis was compared to the cleanliness of blastoderm from previous study (Figure 2.3b).

3.1.2. Single cell preparation for flow cytometry analysis

Different techniques of blastoderm dissociation were used in current study to select the appropriate method that will reduce cellular debris and preserve cell integrity. Majority of these techniques involved tried and error methods after techniques previously used in other studies did not work (Figures 2.3c and 2.3d). Once the appropriate technique was decided, the $PBS^{-/-}$ around the blastoderms was removed and replaced with 50 µl, 37°C pre-warmed commercially available 0.25% trypsin/0.04% EDTA (w/v) solution (Petitte et al., 1990). The blastoderms were incubated for 10 min and pipetted up and down periodically (15x) at room temperature (RT). The samples were incubated for another 10 min at 37°C and repeatedly aspirated again. Fetal calf serum (FCS) was added to stop the action of trypsin. All samples were placed on ice for 2 min and centrifuged for 10 min at 105 xg at RT. Cells were washed in $PBS^{-/-}$ solution at 105xg at RT for 10 min and the supernatant discarded. These centrifugation steps were sufficient to bring down the majority of cells (over 95%). This was tested by placing a sample of the clear solution on a slide and placing it under a light microscope. The cell pellet was resuspended in 500 µl 1X annexin binding buffer (ABB). The concentration of cells in the final suspension was determined on a hemocytometer. An approximately 2.0 x 10^6 cells/ml suspension (determined on a hemacytometer) was resuspended in 400 µl volume, divided into four tubes corresponding to negative control (annex V-/PI-), annexin V-FITC positive control (annex V+/PI-), PI positive control (annex V-/PI+) and experimental sample (annex V+/PI+) groups. The samples were placed on ice and stained according to the labeling and

then analyzed on a flow cytometer (BD FACScan, Becton Dickinson, San Jose, CA) with the following minor modifications: the negative control and PI positive control volumes were increased to 1 ml with 1x ABB and placed on ice. An aliquot of 5 µl each of annexin V fluorescent dye was added to the annexin V positive control and the experimental sample and incubated for 15 min in RT. The contents of these two samples were increased to 1 ml with ABB after incubation. Afterwards, 2 µl each of 10 mg/ml PI dye was added to the experimental sample and PI positive control and incubated for 15 min on ice. After incubation, the four samples were fixed in 1% formaldehyde at 4^{o}C overnight and subjected to flow cytometer data acquisition and analysis (BD FACScan). Prior to analysis, the cells were stained with a dye, deep red fluorescing agent (DRAQ5, Biostatus Ltd., Leicestershire, UK) which is a novel dye with a high affinity for DNA that rapidly penetrates the plasma membrane of both viable and non-viable cells (Smith et al., 1999). Addition of DRAQ5 allowed determination of the degree of nuclear fragmentation as it stains the DNA and reveals distribution of nuclear materials in the cytoplasm.

3.2. Flow cytometer data acquisition and analysis

The flow cytometer used was a bench top, single excitation blue argon laser at 488 nm (BD FACScan, Becton Dickinson, San Jose, CA) capable of measuring parameters such as forward scatter (FSC-H), side scatter (SSC-H), and fluorescence parameters (FL1 H, FL2 H and FL3 H). Fluorescence emissions were collected at 515-545 nm (green) for annexin V and 564-604 nm (red) for PI.

Data were analyzed with Cell Quest software (BD Cell Quest Pro software, Becton, Dickinson and Co., San Jose, CA) and the doublet discrimination module was used to determine the degree of doublets in the cell suspension (Figure 2.4). The threshold of FSC-H was placed at 52 V while the flow cytometer voltages were adjusted to linear mode. Data were collected on 10,000 cells per cell sample. The total population of cells was collected on forward scatter: FSC-H (cell size) versus side scatter: SSC-H (cell complexity) plot (Figure 2.5a). A sample of the cells gated from FSC-H versus SSC-H was separated according to the fluorescent staining ability of annexin V versus PI (Figure 2.5b). The population of cells in each quadrant was expressed as a percentage of the total gated cells.

3.3. Imagestream multispectral flow cytometer analysis

The cell suspensions were further analyzed on an ImageStream multispectral flow cytometer (Amnis Corporation, 2505 Third Avenue, Suite 210, Seattle, WA) to confirm the occurrence of apoptosis. An ImageStream multispectral imaging flow cytometer works similar to classical flow cytometers except that it also allows visualization of individual cells passing through its flow chamber. The snapshots of each cell provided additional information of cellular morphology and spatial distribution. A unique disadvantage in using classical flow cytometer (FACScan) was that it lacked the function to discriminate and sort out clumps from the cell suspension. This discrepancy was resolved after analyzing the cell suspension in the ImageStream multispectral flow cytometer (Figure 2.6). This machine made it possible to gate around only single cells for

analysis which is more refined compared to single cell gating in some conventional flow cytometers. However, the results with ImageStream multispectral flow cytometer were not different from those obtained using the classical flow cytometer (FACScan). This could indicate that the cells not used in the ImageStream multispectral flow cytometer analysis were bigger cells rather than clumps in the cell suspension and therefore there were little or no clumps in the cell suspension.

3.4. Comparison of cell death between egg storage treatments

3.4.1. Layer eggs

In the second phase of this study eggs were collected from the same layer flocks and stored for 4 d and 14 d. The techniques of blastoderm isolation and cell separation were used to dissociate blastoderms (40 per treatment) in trypsin/EDTA solution into individual cells, stained with the same fluorescent dyes and subjected to flow cytometer data acquisition as described previously. However, the isolation of blastoderms from 14 d stored eggs was more challenging as the blastoderms were more fragile and required gentler handling than blastoderms from 4 d and unstored eggs. Nevertheless, only intact blastoderms that did not break down during the isolation stage to obtain dissociated cells were used. Each experiment was replicated 4 times. The percentages of live cells, early apoptotic cells, late apoptotic/necrotic cells and necrotic cells were obtained between the two storage durations (4 and 14 d) as well unstored eggs. Blastoderms from unstored eggs were also examined using

BD FACScan and ImageStream multispectral flow cytometer to determine the characteristics of chicken cells soon after oviposition.

3.4.2. Broiler eggs

In the third phase of the study, the techniques developed with layer eggs were used to separate blastoderms from broiler breeder eggs into individual cells. In this study, three batches of freshly laid fertile hatching eggs (60 per trial) were obtained from 47 wk old Ross 308 experimental flocks as mentioned above. Each set of eggs was divided into two groups and stored at 16-18^{o}C and 70-80% RH for 4 d while the other batch was stored for 14 d under the same temperature and RH. After storage, whole blastoderms isolated from the eggs without damaged area pellucida and area opaca were separated into individual cells after treatment with trypsin/EDTA and subjected to analysis on a flow cytometer (BD FACScan) as shown above.

3.5. Proving evidence of apoptosis and necrosis

The morphology of the isolated blastodermal cells from both layer and broiler breeder eggs were next examined using the ImageStream multispectral flow cytometer for evidence of apoptotic and necrotic cell death. Because the ImageStream multispectral flow cytometer can allow for increased spatial resolution and examination of morphological changes, blastodermal cells from all the egg storage treatments were applied for the purposes of comparing data. It was observed that the results were similar to the measurements from classical flow

cytometer. Figure 2.6 shows the process of separating blastodermal cells into viable, early apoptotic, necrotic, and late apoptotic-necrotic cells under the ImageStream multispectral flow cytometer. The results also provide evidence of the occurrence of apoptosis and necrosis according to differential absorption of either annexin V or PI dye (Figure 2.6c).

4. MOLECULAR ANALYSIS

4.1. Isolation of broiler embryos and RNA extraction

Hatching eggs were obtained from Ross 308 flocks as reported above, divided into two groups, and stored for 4 d and 14 d for gene expression analysis under the same conditions mentioned above. Upon storage, 40 eggs per storage treatment were used to isolate intact blastoderms using RNase free water, pooled together and snapped frozen in liquid nitrogen for further studies. Because embryonic metabolism was not measured at the blastodermal level but after incubation, the expression of apoptosis genes were also studied for incubated eggs after storage for 4 d and 14 d. This was to help assess what impact the incubation conditions will have on the development of embryos from stored eggs under different conditions. Therefore, 36 eggs from each storage treatment were incubated for 6 d at 37.5^{o}C and 56-57% RH. The 6 d of incubation was chosen because at this time point, all embryonic morphology was practically present and RNA extracted was a representative sample of the entire embryo body. The 6 d old embryos were harvested under sterile conditions for RNA extraction. Total RNA was extracted from both blastoderms and 6 d old embryos using TRIzol

(Invitrogen Canada Inc., Burlington, ON) and Precellys lysing kits (Cayman Chemical, Michigan) (Appendix 2). The messenger RNA was then reverse transcribed into cDNA using superscript II reverse transcriptase (Invitrogen Canada Inc., Burlington, ON) and Oligo-dT primers (Invitrogen Canada Inc., Burlington, ON) (Appendix 3). The cDNA was used as a template for qRT-PCR analysis.

4.2. Real time quantitative polymerase chain reaction

4.2.1. Selection of apoptosis genes and bioinformatics

Five known genes associated with apoptosis mechanisms were selected for gene expression analysis: pro-apoptotic associated genes: *Bcl-2*-associated X gene *(Bax)*, *Bcl-2* homologous antagonist/killer gene *(Bak)*, *Bcl-2*-related ovarian killer gene *(Bok)* and anti-apoptotic associated genes: B-cell lymphoma 2 gene *(Bcl-2)* and B-cell lymphoma xL gene *(Bcl-xL)*. Genes were selected based on their role in apoptosis, sequence availability in NCBI (National Center for Biotechnology Information) and confirmed with Ensembl database. The chicken sequences of these genes were obtained from NCBI database and used to designed primers. To confirm the existence of genes, the protein sequence of the selected genes were blasted and reverse blasted using NCBI (T-BLAST-N) and Ensembl bioinformatics tools against mouse, zebrafish, bovine or turkey protein sequences of the same gene to established sequence identity and to ensure that the particular gene was not occurring by chance. The protein sequence of each gene was reverse blasted for the chicken sequence. Once the genes were confirmed using the

bioinformatics tools, DNA sequences specific to the selected apoptosis genes were used to design forward and reverse primers from the 5' end of the sequence to the 3' end on both the sense and antisense sequences (Applied Biosystems primer express 3.0).

4.2.2. Selection of housekeeping genes

The expression levels of all candidate genes were normalized using the average Ct values of three housekeeping genes: *HPRT* (Hypoxanthine-guanine phosphoribosyltransferase), *β*-actin (*Beta*-actin) and *UBQ* (Ubiquitin) which were found to be stably expressed based on their coefficient of variations (CV ≤ 6 %) (Chen et al., 2010). In this thesis 3 out of 5 housekeeping genes that had been successfully used in the development of lipopolysaccharide inflammation model in chickens (*β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phophoribosyl-transferase (HPRT), ubiquitin (UBQ) and glucose-6-phosphate dehydrogenase (G6PDH*) (Boever et al., 2008) were used as normalization genes. In their study, *β*-actin appeared to be the most stable single gene. In addition to *β*-actin, the authors discovered that the most stable combination was *G6PDH* and *UBQ* based on better correlation coefficients (Vandesompele et al., 2002). However, it was decided that the average of all the 3 genes (*β-actin, HPRT and UBQ*) based on non-significant difference between the threshold cycle (Ct) values of 4 d and 14 d treatment groups will be used (Boever et al., 2008). In addition, when the Ct values were statistically different, gene combination with lowest coefficient of variation (CV) between the two storage

treatments were used as normalization genes. Previous study had shown that when the CV is lower than 10%, the gene is considerably stable and can be used as a housekeeping gene (Chen et al., 2010).

4.2.3. Primer design

Sequence of 8 genes (3 housekeeping and 5 candidate genes) of chicken origin were obtained from PUBMed (www.ncbi.nlm.nih.gov/sites/entrez) and confirmed from ensemble (http://uswest.ensembl.org/index.html) to determine exons and introns position. The exons region is required to design primers because exon regions correspond to the protein coding regions of a gene unlike the intron regions. The sequences were used to design primers for qRT-PCR analysis using Tagman MBG setting on Primer express 3.0 software (Applied Biosystems, Streetsville, ON). Probes were designed as part of the Tagman method but were not used in the current study because the specific expressions of the genes were not kown; therefore the use of the probes will be expensive if experiments do not work. Each primer set was designed to amplify a qRT-PCR product size (amplicon length) between 50 to 150 bp (Table 2.1). The suitability of the chosen primer sets were determined by the presence of single peaks on dissociation curves generated during qRT-PCR analysis.

4.2.4. Verify suitability of housekeeping genes

The expression of the housekeeping genes (based on different pairs of primers) were first verified to determine their stability levels as normalization

genes. The best primer set was chosen based on the tightness of individual curves (triplicate sample) and closeness of curves between the two treatments (4 d versus 14 d) for further studies to determine PCR efficiency and gene expression profile between storage treatments. Each experiment was repeated and all Ct values obtained were analyzed statistically ($P \leq 0.05$). The Ct value is defined as the threshold cycle number in which there is a significant increase in the amount of PCR product. Relative abundance of mRNA was determined by interpolation from standard curves to create linear values for each sample.

4.2.5. qRT-PCR analysis of target genes

Quantitative real time PCR (qRT-PCR) analysis was performed using SYBR Green chemistry with a 7500 Fast Real-Time PCR System (Applied Biosystems, Streetsville, ON). The reaction solution contained 5µl SYBR Green mastermix, 2.5µl cDNA and 2.5µl primer set (3.2µM) for each sample and the reaction was performed using the following program: 95°C for 2 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min, and a dissociation stage of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 15 sec in a 96-well reaction plate. For each gene, qRT-PCR was performed in triplicate and a negative control was included. A dilution series of cDNA template from each egg storage treatment were obtained. The results were graphed using the Applied Biosystems 7500 Fast Real-Time PCR System software which generated linear lines and equations. The slope of each line was used to determine the efficiency of the PCR experiment and any reaction with efficiency above 85% was used for further analysis.

Relative abundance of mRNA or fold change (cDNA) was determined at the exponential phase as the difference between the threshold cycle values (Ct) measured in egg storage d 14 and d 4 and normalized with the average Ct value of the housekeeping genes (*β-actin, HPRT and UBQ*) (Equation 4).

$$\text{Fold change} = 2^{-\Delta\Delta Ct} \quad [5]$$

Where $\Delta\Delta Ct = \Delta Ct$ (14 d) – ΔCt (4 d)

ΔCt = Ct (14 or 4 d of target gene) – Ct (housekeeping gene)

4.2.6. Gene transcript sizes

The transcript sizes of genes amplified by qRT-PCR procedure were determined by agarose gel electrophoresis (2% gel; 80V for 4 h) followed by ethidium bromide staining (10 mg/ml, Gibco BRL). DNA was then visualized by ultraviolet examination (Syngene, Synoptics Ltd., Cambridge, UK)). The size of DNA was compared to that of standard molecular markers.

5. GENE EXPRESSION IN TURKEY EMBRYOS

A gene expression study has been repeated in turkey embryos obtained from 6 d incubated eggs (Appendix 4).

6. STATISTICAL ANALYSIS

Data obtained from all experiments were analyzed using the PROC MIXED MODEL procedure of SAS at $P \leq 0.05$ (SAS Institute, 2002-2003).

Where significant differences were observed, the least squares means were separated by PDIFF. Embryonic metabolism between genetic strains and also flock age data were analyzed as 2–way randomized design because the eggs were obtained from different farms; all other data were randomized by complete block design. Because there was significant difference between initial egg weight before storage in the broiler breeder eggs used to investigate metabolic difference, all parameters investigated were analysed by using egg weight before storage as a covariance to eliminate the effect of egg weight and assess only the effect of egg storage.

Table 2.1. Selected genes and primer used in this study

Gene	Accession number	Type	Primer sequence[1]	qRT-PCR Product size (bp)
Bak	NM_001030920	F	GCAGCCCACCAAGGAGAA	70
		R	AATGCCGCTGTCGAACAAG	
Bax	XM_422067	F	TCCTCATCGCCATGCTCAT	69
		R	CCTTGGTCTGGAAGCAGAAGA	
Bok	NM_204706	F	GAATACATTCGCCCCAACGT	62
		R	CGAGTGCAGCGAGATGTTCA	
Bcl-2	NM_205339	F	GGCACCTGCACAACTGGAT	63
		R	TGCCGTACAATTCCACAAAGG	
Bcl-xL	NM_001025304	F	ACGGCGTACCAGAGCTTTGA	63
		R	CCCCCAGTTCACACCATCA	
HPRT	AJ132697	F	TTTGCACTATGACTCTACCGACTATTG	70
		R	AGACTCCGGAGCTCACAAACAG	
B-actin	L08165	F	AGCAAGCAGGAGTACGATGAATC	64
		R	ACAGTCCGGTTTAGAAGCATTTG	
UBQ	M11100	F	GCTATTAGTTGTTGTCAGCAGTCTTGT	78
		R	GACATTAGAAAGACTACAGTGCAACACA	

[1]Gene sequences were obtained from NCBI and Ensembl databases. The F and R mean forward and reverse primers respectively in 5' to 3' directions on DNA gene sequence.

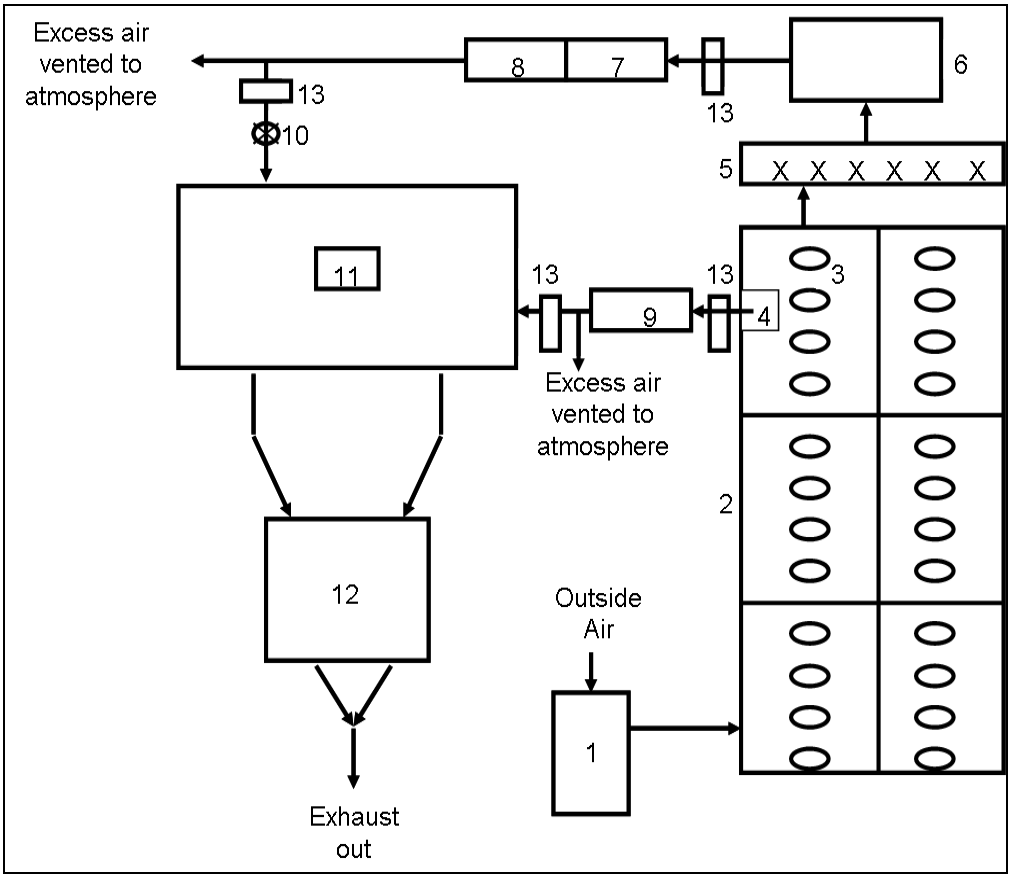

Figure 2.1. The avian embryonic metabolism system use to measure embryonic oxygen consumption and carbon dioxide production. The avian embryo metabolism system of, (1) Premixing air chamber, (2) Incubator, (3) Metabolic chambers, (4) Reference air from incubator, (5) Air sampling valves, (6) Carbon dioxide analyzer, (7) Sampling air pump, (8) Sampling air pump controller, (9) Reference air pump, (10) Needle valve to balance differential pressure between sample (S) and reference (R) O_2 sensors, (11) Differential oxygen analyzer, (12) Dual flow pumps draw air through the DOX at a rate of 25 ml/min, (13) Moisture absorbing magnesium perchlorate.

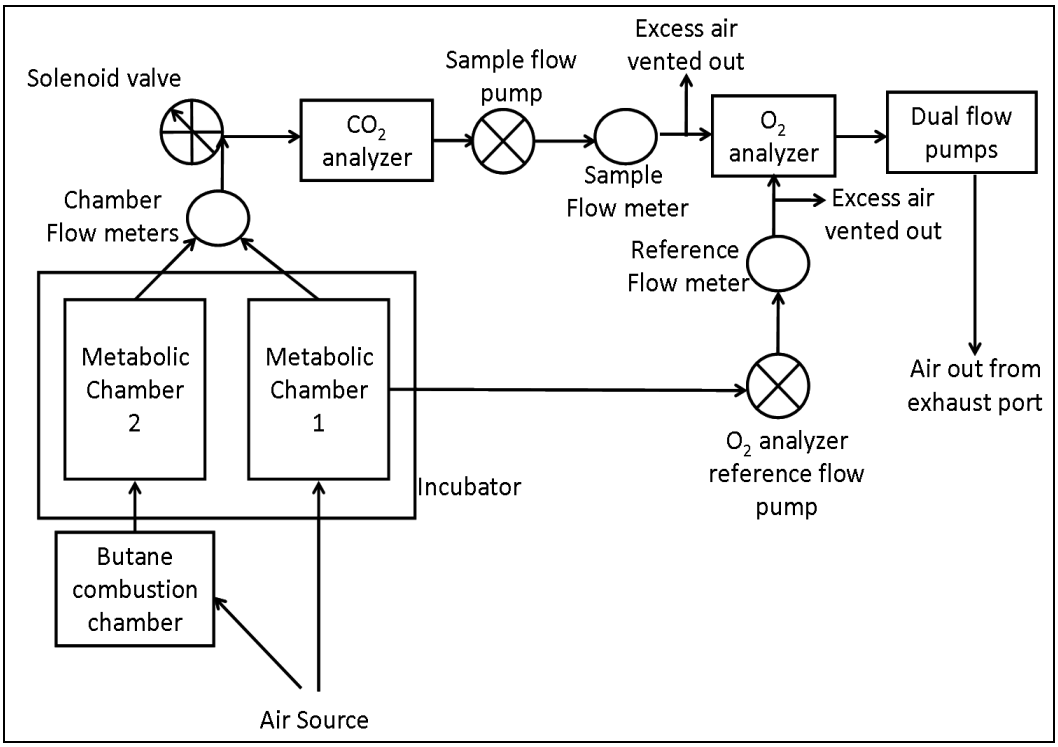

Figure 2.2. Schematic diagram representing the calorimetric system using butane combustion gases to validate the accuracy of O_2 and CO_2 analyzers and chamber flow meters**.**

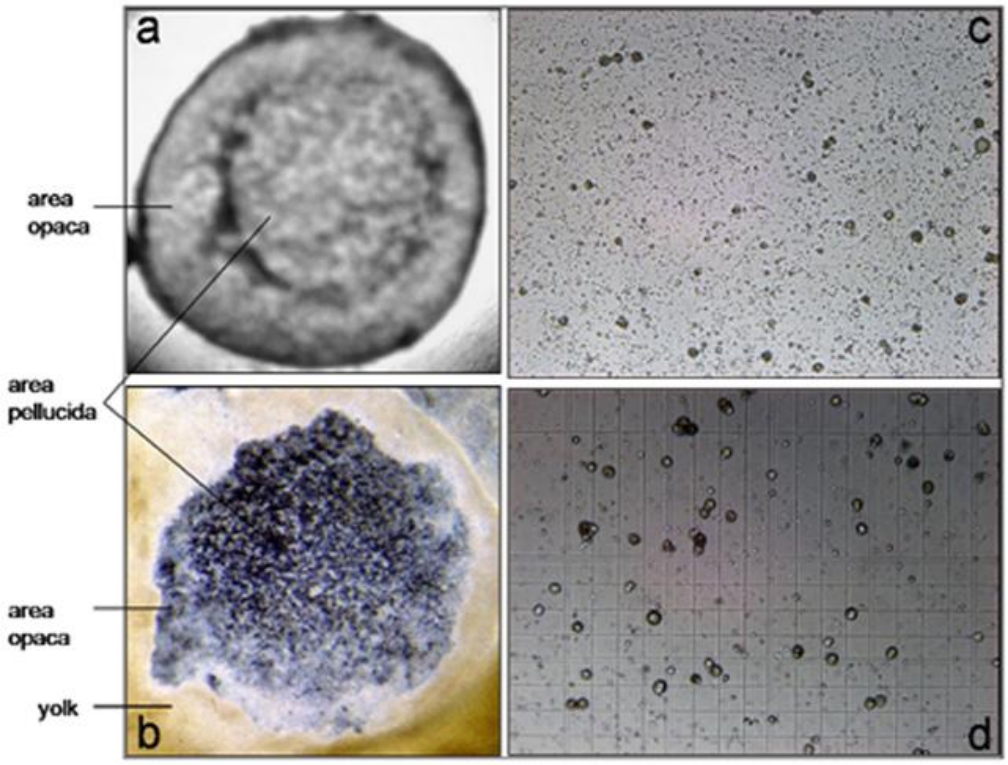

Figure 2.3. Chicken blastoderm isolated and dissociated into single cells. (A) Blastoderms were harvested from egg yolk surface by modifying technique previously used by Petitte et al. (1990). In our modification technique, the blastoderm was cleaned by drop-wise release of $PBS^{-/-}$ unto the surface of the blastoderm to wash yolk fragments off. The two defined areas: area pellucida (inner layer) and area opaca (periphery) of Eyal-Giladi and Kicked (1976) stage XI blastoderm specifications were evident using the modified technique. (B) Similar blastoderm isolated by Fasenko et al. (2009) using the previous technique (Petitte et al., 1990). Area opaca is still covered by yolk. (C) Microscopic observation (magnification: 20x) of blastoderms dissociated into single cells using previous method by Petitte et al. (1990). The cell suspension contained more debris with background grid lines completely covered. (D) Single cells prepared from currently developed dissociation technique. In this technique blastoderms were repeated aspirated for a specified number of times at two time point with 20 min incubation time in trypsin/EDTA solution. The cell suspension had less debris compared to previous method. Cell sizes were calculated based on grid lines; bigger square measures 1mm by 1mm while small square measures 250 by 250 µm.

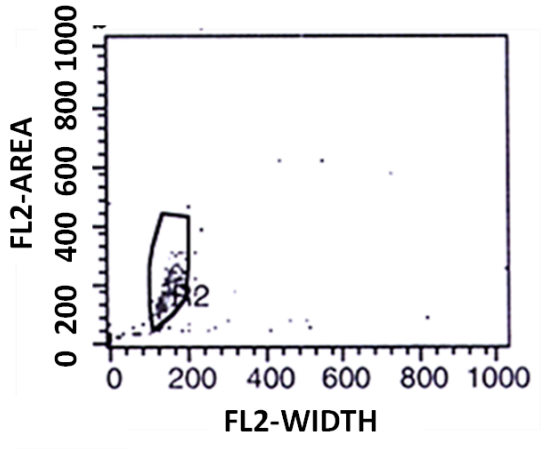

Figure 2.4. Doublet discriminated illustrated by plotting the area of cells with the width of the cells. Cells with both higher pulse area and width mean they have more DNA just like cells in dividing mode and therefore are plotted as doublets. All the cells indicated are single cells with almost no sign of doublets in the sample. These cells are from chicken embryos dissociated using the current dissociation protocol.

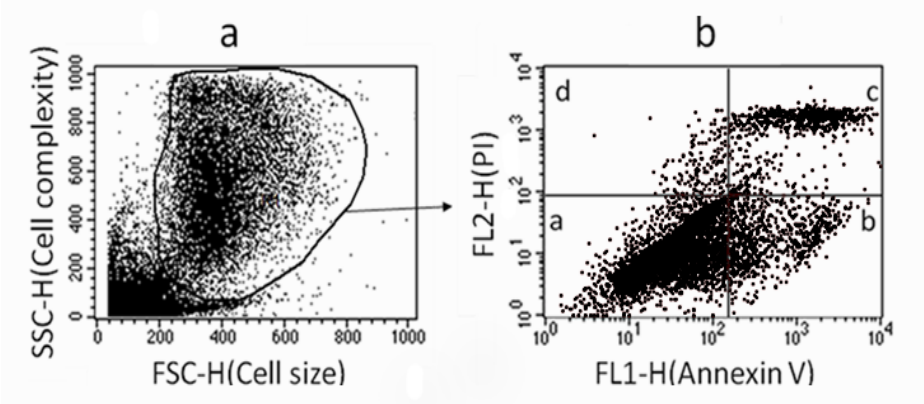

Figure 2.5. Quantitative analysis of blastodermal cell suspensions using flow cytometry method. Isolated blastodermal cells were stained with annexin V-FITC and PI, and fixed with 1% formaldehyde. Cell were then washed with $PBS^{-/-}$ and analyzed on a flow cytometer (BD FACScan). (A) Total cell population cells recorded according cell size (forward scatter) and internal complexity (side scatter) and showing event distribution in a representative cell suspension prepared from chicken eggs stored for 4 d, and (b) Gated cell population was separated into a) Annex V-/PI- cells (live), b) Annex V+/PI- cells (early apoptosis), c) Annex V+/PI+ cells (late apoptosis or necrosis), and d) Annex V-/PI+ cells (necrotic cells) according to cellular absorption of annexin V-FITC and PI. Cell suspension from unstored eggs and eggs stored for 14 d were also separated in the same manner.

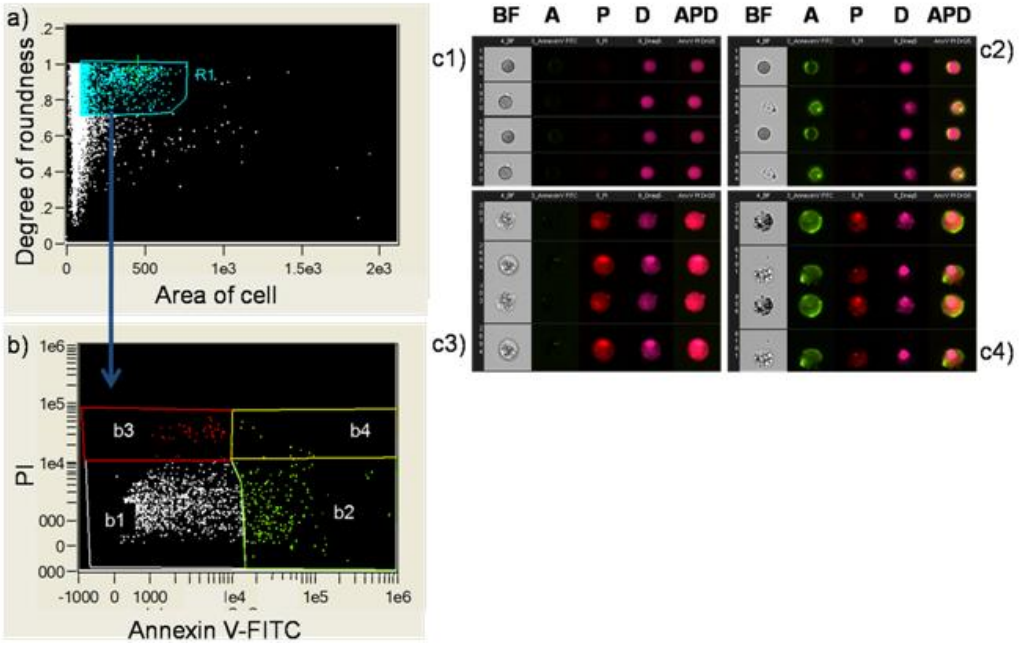

Figure 2.6. Flow cytometry based cellular classification according to fluorescent absorption and morphological examination of blastodermal cells using ImageStream multispectral flow cytometer. (A) Data acquisition and gating of single cells is based on the cell surface and aspect ratio of cell height and area. (B) Representative analysis of gated cells to show procedure for classification of blastodermal cells into: b1) live (AnnexV- / PI-), b2) early apoptotic (AnnexV+ / PI-), b3) necrotic cells (AnnexV- / PI+), and b4) late apoptotic/necrotic (AnnexV+ / PI+). (C) Morphological examination of blastodermal cells classified into: c1) live (AnnexV- / PI-), c2) early apoptotic (AnnexV+ / PI-), c3) necrotic cells (AnnexV- / PI+), and c4) late apoptotic/necrotic (AnnexV+ / PI+). Abbreviations indicate bright field (BF), annexin V-FITC (A), propidium iodide or PI (P), DRAQ5 (D) and an overlay of annexin V-FITC, PI, and DRAQ5 (APD).

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CHAPTER 3: ASSESSMENT OF AN INDIRECT CALORIMETRIC SYSTEM FOR MEASUREMENT OF AVIAN EMBRYONIC METABOLISM[1]

1. INTRODUCTION

Accurate measurement of embryonic metabolism and respiratory quotient (RQ) of birds has been a subject of interest spanning over decades (Pearson et al., 2002). Indirect calorimetry utilizes the oxygen (O_2) consumption and the carbon dioxide (CO_2) production values to calculate heat production and RQ (Kleiber, 1987; Hamidu et al., 2007). The RQ which is the ratio of CO_2 production to O_2 consumption (McClave et al., 2003) indicates the nutrients (carbohydrates, proteins or lipids) being metabolized (Gefen and Ar, 2001). The avian egg and the embryo developing within represent a closed system in which the entire supply of nutrients, including vitamins and minerals necessary to support complete embryonic development, is deposited by the hen into the egg at the time of its formation (Richards, 1996). Although the avian egg has been described as a closed system in other studies (Berg et al., 1999) it can exchange gases with its environment. However, the embryo occupies a defined environment where there dictates the rate of exchange of gases between the egg and its environment (Rahn et al., 1979). It is very important that any experimental apparatus that is used to study the energetics of the embryo does not have a negative effect on the embryo.

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[1]This portion of the thesis has been published: Hamidu, J. A., J. J. R. Feddes, C. A. Ouellette and G. M. Fasenko. 2010. Canadian Biosystems Engineering 52 (4): 9–14.

J. A. Hamidu was involved in the collection data, analysis, and writing of the manuscript.

A number of researchers have attempted to measure the process of gaseous exchange in avian embryos, along with the accuracy of the methods (Monge et al., 2000; Mortola and Labbe, 2005; Christensen et al., 2007). In most of the methods, only a single egg was monitored at a time and the number of measurements that could be obtained during the experimental period was limited (Sato et al., 2006). Also, some of the methods could not monitor the embryo up until the end of incubation (Carey et al., 1989). Because some of the methods involved the handling of eggs throughout the experiment, accurate measurements of the O_2 consumption and CO_2 production rates were difficult to report. In addition, measurements were also tedious as the researchers had to constantly observe the operation of the respiration equipment (Vleck and Kenagy, 1980). In the last decade, indirect calorimetry has been used to continuously measure embryo CO_2 production during the incubation period. With this method, embryonic heat production was calculated from the measured CO_2 production and reported RQ values (Vleck et al., 1980; O'Dea et al,. 2004; Segura et al., 2006). From the CO_2 production values and RQ values, the O_2 consumption values were calculated (O'Dea et al., 2004). A method developed by Segura et al. (2006) involved incubating chicken eggs individually in 24 1-L metabolic chambers inside an existing incubator. Each chamber was sampled for 2.5 min every 1 h for CO_2 production.

Sato et al. (2006) measured a chicken embryo's O_2 consumption and CO_2 production but could only do so after 12 d of incubation because concentrations of these gases are very small during the early periods of incubation. They also

reported an RQ value of 0.71 over the incubation period studied. Because the RQ of lipids is 0.7 (Krogh and Lindhard, 1920) the RQ value reported by Sato et al. (2006) suggested that chicken embryos were metabolizing almost exclusively lipids after 12 d of incubation. Hamidu et al. (2007) reported different RQ values during a 21-d incubation period of chicken eggs. They reported that the RQ of chicken embryos during the first 10 d was approximately 1.00 suggesting that the embryos were metabolizing carbohydrate substrates. Afterwards, the RQ value began to decrease; by d 21 the RQ value was 0.63, which suggested that the embryo was actively involved in lipid metabolism. In order to accurately obtain RQ data, both the O_2 and CO_2 concentrations must be measured frequently over the incubation period.

The objective of this study in the current thesis was to determine the accuracy of a custom made calorimetric system over the entire incubation period in order to use it to assess the metabolism of avian embryos. This included the performance of the gas monitoring equipment and the airflow rate measuring devices. The hypothesis of the study was that the RQ values obtained from this system would not be significantly different from the theoretical RQ value of a burning butane source at three metabolic chamber air flow rates.

2. RESULTS AND DISCUSSION

2.1. Accuracy of a gas combustion system

The combustion of butane is the following: $C_4H_{I0} + 6.5\ O_2 = 4\ CO_2 + 5\ H_2O$), 1 mol of butane is equivalent to 6.5 mol of O_2 and the RQ is 0.62 (Nunn et

al. 1989). The mean RQ value using the current calorimetric system was 0.64 ± 0.004 (Table 3.1). The calculated RQ was 3% higher than the theoretical RQ of butane and the standard deviation was 0.019 (n=30). However, there was a small mean but significant difference (0.06) in RQ ($p < 0.05$) between the theoretical and the stoichiometrical calculations (RQ = 0.64). Though the standard error and the standard deviation of the mean were very small, the actual mean difference between the theoretical and current value was also small. Since the two RQ values were only 3% different from each other, the performance of the calorimetry system is acceptable. These results are consistent with those of a similar study where butane combustion resulted in RQ measurement 5% lower than the theoretical RQ value of butane but that study was considered accurate (Forsberg et al., 1986). The minimum RQ value measured (0.62) and the maximum RQ value (0.68) differed by 0.06. While the real difference is small, the standard deviation and standard errors were also very small. Table 3.1 also shows that the real time flow rate measurements were within the expected values of 75, 100 and 300 ml/min (±5 ml/min). The metabolism system as described in chapter II (Figure 2.1) can thus be used to measure accurate O_2 and CO_2 concentrations (or O_2 consumption and CO_2 production rates) during avian embryo respiration.

Accurate O_2 consumption and CO_2 production rates data that rely on reported RQ have been a subject of concern especially when small exchange rates occur during the first 7 d in the development of a chicken embryo (Hamidu et al., 2007). Our ability to measure different concentrations of O_2 and CO_2 during butane combustion which resulted in consistent gas exchange rates have given

enough confidence in the use of the calorimetry system to measure avian O_2 consumption and CO_2 production during the first wk of incubation. The data confirm that the results reported in previous studies using this system are reliable (Hamidu et al., 2007). The O_2 consumption and CO_2 production data presented in Table 2.1 were similar to those observed in previous experiments. In our current study, the O_2 exchange rates varied between 8720 and 9240 µl/h, whereas, CO_2 exchange rate values ranged between 5700 and 5920 µl/h using the butane combustion system. In turkey experiment conducted as part of this thesis, embryonic CO_2 exchange rates reached between 5,000 to 7,000 µl/h by 15 d of incubation and reached over 43,000 µl/h by 28 d of incubation when the embryos hatch. This study will be highlighted in detailed in chapter 1V of this thesis. Also, embryonic O_2 exchange rates in the turkey embryos peaked to about 8,000µl/h in 15 d and reached over 60,000 µl/h by 28 d of incubation. Similarly results in broiler breeders show that embryonic CO_2 (~6000 µl/d) and O_2 (~8000 µl/d) exchange rates were reached at d-12 of incubation. By 21 d of incubation when the embryos were hatching CO_2 and O_2 exchange rates reached about 34,000 µL/h and 50,000 µl/h, respectively (Hamidu et al., 2007).

In recent years, emphasis has been placed on measurements of CO_2 production in the estimation of embryonic heat production and RQ (O'Dea et al., 2004; Segura et al., 2006). One reason is the availability of improved instrumentation, with current CO_2 analyzers being approximately 100 times more sensitive than typical O_2 analyzers (Walsberg and Wolf, 1995). However, estimating metabolic energy production from CO_2 measurement alone may lead to

substantially larger errors than those obtained from measurements of both O_2 consumption and CO_2 production. This could be due to a number of assumptions including the substrate that an embryo is metabolizing as well as the RQ value assumed to calculate O_2 consumption from CO_2 production data (Walsberg and Wolf, 1995). As such the validation of the current calorimetric system, which has an O_2 analyzer that has an accuracy varying within ±10 ppm will produce reliable gas exchange and heat production data even though statistically, the theoretical and calculated RQ of butane were significantly different.

2.2. Accuracy or precision of calorimtry system

One of the disadvantages of using the current calorimetry system is the precision at which it measures gases. It has been proven using the RQ that it can recorded data at about 97% accuracy. However, using the same system to measure avian respiration showed that the data recorded during the first 3 days was still fluctuating especially using the DOX. It is important that in future, the precision at which this analyzer (DOX) records data is also validated or more sensitive sensors are used in the DOX to obtain data during the first 3 days of the experiment. This help to assess the embryos metabolic activity throughout incubation.

2.3. Summary and conclusions

Calibration of the CO_2 and O_2 analyzers and the flow meters are essential to develop confidence in measuring embryo gas exchange and energy production.

In this study, butane combustion was found to simulate the respiration of an organism inside a metabolic chamber with O_2 and CO_2 exchange rates that were typical of avian embryo CO_2 production and O_2 consumption rates. By validating the performance of the CO_2 and O_2 analyzers with butane (theoretical RQ value of 0.62) it has been confirmed that the current avian calorimetric system as previously used, measures O_2 and CO_2 concentrations reliably. In the current study RQ calculated (0.64) was only 3% off from the theoretical RQ of butane (0.62). It can be conclude that O_2 consumption and CO_2 production rates or exchange rates, and flow rates measured in previous (Hamidu et al., 2007) and ongoing (Hamidu et al., in press) studies with chicken and turkey embryos respectively were measured with sufficient accuracy.

Table 3.1. Respiratory quotient from individual butane combustion measurements

ID	Flow rate[1] (ml/min)	CO_2 production rate[2] (µL/h)	O_2 consumption rate[2] (µl/h)	Respiratory quotient[3]
1	73	5,920	8,720	0.68
2	74	5,880	8,790	0.67
3	75	5,910	8,740	0.68
4	75	5,750	8,980	0.64
5	76	5,770	8,880	0.65
6	76	5,820	9,010	0.65
7	95	5,670	8,900	0.64
8	95	5,720	8,960	0.64
9	95	5,670	9,200	0.62
10	95	5,760	8,840	0.65
11	95	5,720	9,030	0.63
12	95	5,740	9,040	0.64
13	95	5,810	8,560	0.68
14	95	5,710	9,110	0.63
15	96	5,730	9,050	0.63
16	96	5,700	9,240	0.62
17	96	5,700	9,240	0.62
18	96	5,810	8,700	0.67
19	96	5,800	8,700	0.67
20	96	5,780	8,950	0.65
21	96	5,830	8,790	0.66
22	96	5,790	9,100	0.64
23	96	5,740	9,230	0.62
24	96	5,740	9,240	0.62
25	298	5,780	9,120	0.63
26	299	5,770	9,190	0.63
27	295	5,860	8,850	0.66
28	301	5,890	8,900	0.66
29	296	5,840	8,970	0.65
30	300	5,850	8,900	0.66

[1] Flow rate monitored at three different levels (75, 100 and 300 ml/min).

[2] Gas exchange rate = O_2 or CO_2 concentration x sample air flow rate/10^6.

[3] Respiratory quotient = CO_2 exchange rate/O_2 exchange rate.

P value < 0.05 (Statistically, theoretical value of butane is lower than measured value).

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CHAPTER 4: EFFECTS OF GENETIC SELECTION AND PARENT FLOCK AGE ON EMBRYONIC METABOLISM[2]

1. INTRODUCTION

There is a lot of evidence to show that genetic selection in turkeys has in the past years resulted in increased breast muscle yield both during the embryonic period (Velleman et al., 2002) and posthatch growth (Velleman et al., 2003). However, the physiological demands or the daily metabolism needs of embryos from turkey strains selected to yield large breast muscles for consumption and their responses to current incubation strategies have not been well understood. Study shows that changes in breast muscle morphology were highly significant when both genetic lines and maternal flock age were considered (Velleman and Nestor, 2005). In four galliform species including turkeys, because embryonic growth rate is normally high, embryos allocated energy in favour of their maintenance costs (Dietz et al., 1998). Additionally, the bigger size of breast muscles of modern turkey strains means that additional energy was diverted for growth and maintenance of the 'metabolically active' breast or pectoral muscle tissues (Bagley and Christensen, 1989).

While most current strains of turkeys have been selected for breast muscle deposition the embryos also grow bigger than their random bred strains

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(Applegate, 2002; Christensen et al., 2001a, 2007, 2008). For this reason there is additional demand on the embryo to metabolize faster to meet the energy requirements for maintenance and growth of demand tissues like muscles and heart muscles (Christensen et al., 2001b). Nevertheless, the egg can provide that much space for the embryo to growth and also enough eggshell pores for gas exchange because of the physical barrier created by the eggshell (Rahn et al., 1979). A recent study showed that while genetic selection has altered embryonic cardiac energy metabolism, the possibility that cardiac failure as it relates to energy metabolism may be a factor in embryonic poult mortality (Christensen et al., 2008). Meanwhile, embryos from turkey strains selected for growth were reported to have different energy metabolism that relied more on gluconeogenesis (Christensen et al., 2008). But it is not clear if the embryonic metabolism of certain turkey strains is higher than others, which could trigger overheating in commercial incubators as has been the case in some broiler breeder strains (O'Dea et al., 2004; Hamidu et al., 2007).

The impact of selection can also be due to incubating eggs from different flock ages. While eggs from younger parent flocks are smaller than eggs from older parent flock ages, under the same incubation conditions embryos from young parents also develop more slowly than embryos from older hens during the last week of incubation (Christensen et al., 2001a; Applegate, 2002). The associated difference in growth pattern can cause embryos from older flocks to reach the plateau stage of O_2 consumption earlier than embryos from young flocks (Christensen et al., 1996). Under this circumstance the embryos from older flocks

may utilize O_2 in their air cell prematurely after internal pipping before they can fully hatch resulting in increased late embryo mortality. In addition, embryos from young flocks may experience longer hatching time which can result in dehydration and death.

Recently, a study has showed that embryonic metabolism of two modern broiler genetic strains and six parent flock ages were different especially during early and late periods of incubation (Hamidu et al., 2007). Interestingly, these two periods of incubation appeared to coincide well with the two periods of increased embryonic mortality during commercial incubation of turkey eggs (Fairchild et al., 2002). The hypothesis is that a follow up study in turkey embryos would show similar metabolic patterns. The knowledge can be used to advise hatchery managers on how to improve turkey incubation and increase hatchability of individual turkey strains or flocks. Therefore, the objectives of the current study were to examine the effects of turkey genetic strain and parent flock age on eggshell conductance or **G** (ability to exchange gas between the egg and the environment) and daily embryonic heat production (**EHP**), often used as an indicator of embryonic metabolism. The daily respiratory quotient (**RQ**) data in turkey embryos were also calculated since such data are only common with chickens.

2. RESULTS AND DISCUSSION

2.1. Eggshell Conductance and Egg Composition

2.1.1. Genetic strain

Between genetic strains the percentage of moisture loss from the eggs did not differ. Eggshell thickness was not different between strains (Table 4.1). The percentage of wet yolk (29.73, 28.40; *P*=0.0182) and wet eggshell (9.15, 8.77; *P*=0.0019) and dry eggshell (9.02, 8.68; *P*=0.0035) were respectively greater in H than N eggs but there were no differences in percentage of dry yolk (17.50, 16.65; *P*=0.1209) between strains (Table 4.2). Nicholas eggs had greater percentage of wet albumen compared to H eggs (Table 4.2). The data on dry weight show that actual yolk nutrient are the same between N and H strains because differences observed in the wet samples were due to moisture. Similar results reported by Siopes (1995) showed a smaller percentage of yolk and a greater percentage of wet albumen in eggs from N hens compared to those of H or British United turkeys. However, the authors reported that the mean percentage of eggshell weight was greatest in eggs from H hens, which was similarly observed in the current study. The G was higher in the N strain compared to the H strain.

2.1.2. Parent flock age

For the breeder parent flock ages, the moisture loss from eggs was significantly higher in O flock than M, P and Y flocks. While moisture loss from M and P flocks was not significantly different, it was higher than that lost by eggs from the Y flock (Table 4.1). Though eggshell thickness was not different

between O (0.397 mm) and P (0.379 mm) flocks, it was greater in eggs from O flock (0.397 mm) compared to eggs from M (0.340 mm) and Y (0.360 mm) (P<0.0001) flocks which did not differ from each other (Table 4.1). Shell thickness did not differ between P and Y flocks. Apart from M flocks measurement that did not fit into a trend, the rest of the eggshell thickness increased with flock age. While it is well acceptable that eggshell thickness decreases with flock age, we cannot exactly explain why the trend appeared to be in opposite direction. Perhaps user to user recordings of the data may have contributed an error. We can also speculate that the flocks may have received more calcium than normal to deposit the eggshells as flocks aged. However, this may not be the case because eggshell weight decreased with flock age. Although there is no research that has compared eggshell thickness between eggs from different parent flock ages in turkeys, Christensen et al. (2006a) reported that thicker eggshells (0.44 mm versus 0.39 mm) improved embryo viability by improving cardiac health at the plateau stage of O_2 consumption (Christensen et al., 2006a, b). Since eggshell thickness increased with flock age, it could have contributed to the daily heavier weight of embryos recorded in the current study (Table 4.1). However, all the eggshells thickness recorded in this study were lower than 0.44 mm and therefore we are not sure if the eggshell thickness in older flocks did contribute anything like improving embryo cardiac physiology that resulted in better embryo weight. However, all eggshell thickness recorded in the current research were lower than 0.44 mm. Eggshell conductance increased as flock age increased.

As was expected the percentage of wet as well as dry yolk was higher in the M and O flocks than Y and P flocks (Table 4.2). The percentage of wet and dry eggshells and wet albumen were significantly higher in eggs from Y and P flocks compared to eggs from M and O flocks (Table 4.2). These findings agree with results from previous studies that show that as the hen's age increases, yolk weight increases but eggshell weight decreases (Applegate and Lilburn, 1996; Ar and Meir, 2002). The significance of bigger yolk to the embryo could be the availability of more nutrients to the developing embryo, while thinner eggshell or smaller eggshell weight will make it easier for the poult to eternally pip during hatching.

2.1.3. Interaction of genetic strain and parent flock age

Initial egg weight (P=0.0344) and G (0.0227) were significantly different between the interaction of genetic strain and flock age (Table 4.1). In general, the initial egg weight increased as the parent flocks aged regardless of genetic strain. Because increases in parent flock age always leads to increase in egg weight (Lewis and Morris, 1998), it can be stated that the higher G as flock age increased was due to the larger eggs which have larger surface area and therefore more eggshell pores rather than eggshell thickness (P=0.2948). Larger eggs or eggs from older flocks will therefore have higher porosity than small eggs or eggs from young flocks. The G was significantly higher in the O flock (26.46) from H strain eggs and O (26.05) and M (25.47) flocks from N but these were not different from each other. The interaction of N strain by Y (16.70) flock had the lowest G, but

this was not different from the interaction of H strain by Y (71.71) or P (17.53) flock. Nevertheless, G generally increased with parent flock age regardless of genetic strain (*P*=0.0227). The higher G values observed as egg size increased indicate that larger eggs have higher eggshell porosity and probably higher gas exchange capacity than smaller eggs. The trend in our results are different from previously reported data showing no significant difference in either initial egg weight or G between the interaction of genetic strain and flock age (Christensen et al., 2001a). However, the author showed that initial egg weight and G were significant between genetics strain or parent flock ages. The current results and that established in previous study show that turkey strains and/or flock ages are different and may not require the same treatment or incubation of their eggs. This is because the eggs with bigger G (N strain) may have higher porosity and gas exchange than H strain. In previous study a lack of differences in broiler egg weight resulted in no differences in G between flock age. This may indicate that G is dependent on egg weight (Hamidu et al., 2007). There were no significant effects of interaction of genetic strain and flock age on egg components (Table 4.2).

2.2. Egg weight, poult weight, and poult characteristics

2.2.1. Genetic strain

There were no significant effects of genetic strain on pipping time, poult weight, poult length and shank length (Table 4.3). In addition, the percentage of

wet and dry YFBM, and the percentage of wet and dry RYS as well as daily embryo weights were not significantly different between genetic strains.

2.2.2. Parent flock age

Pipping time, poult weight (Table 4.3), poult length, shank length, and percentage of wet and dry yolk sac (Table 4.4) all increased as flock age increased. Conversely, percent wet YFBM decreased with increasing parent flock age. The percentage of dry YFBM was significantly higher in poults from O, P and Y flocks compared to poults from the M flock (Table 4.4). However, it is unclear why the percentage of dry YFBM significantly dropped in M flock. The greater wet YFBM observed in Y and P flocks compared to M and O flocks may indicate that poult weight at hatch as well as the YFBM should be used as proper indicators of poult quality. This is because YFBM may determine the meat yield of the final broiler body weight. However, a different observation was made in previous study where poult weight at younger age was higher in older parent flocks compared to young flocks but the body weight diminished by 63 d post hatch (Schaefer et al., 2002). However, in our current study, we did not grow out the poults and therefore cannot assess body weight at market age. Applegate and Lilburn (1996) reported that RYS weight (DM) increases with parent flock age. This agrees with the current findings.

Though daily embryo weight did not differ between genetic strains, daily embryo weight was higher in O versus Y flock from 4 to 28 d of incubation (Table 4.5). With the exception of 5 and 20 d of incubation, daily embryo weight

was significantly higher in the M flock compared to the Y flock on all days of incubation. Daily embryo weights were statistically greater in P flock compared to Y flock on all days of incubation except 4 to 7, 11, 12, 20, 21 and 26 d of incubation. Generally the daily embryo weight increased with flock age. Daily embryo weight measured from 5 to 26 d of incubation in Bronze turkey eggs (Insko and Lyons, 1933) were in general lighter compared to embryos from the Y flock which had lower BW in the current study. These results show that embryos of modern genetically selected turkey strains have greater body BW than strains available in 1933. The current data on embryo weight could be very important because, since 1993, no research has consistently measured turkey embryo weight on a daily basis. The data also show how embryos from modern turkey genetic strains are different from strains that existed 75 year ago.

2.2.3. Interaction of genetic strain and parent flock age

Egg weights at setting and at transfer were significantly different between the interaction of genetic strain and flock age. Both egg weights increased with flock age regardless of genetic strain (Table 4.3). These were expected because initial egg weight increases with flock age. The interaction of genetic strain and flock age did not affect pipping time, but did affect hatching time (Table 4.3). The hatching time increased with increasing flock age from Y to M flocks with respect to N strain. But the interaction of N strain by O flock was not different from N strain by P flock, while N strain by Y flock did not differ from N strain by P flock. In the H strain, hatching time did not differ between flock ages. Christensen

et al. (2001a) reported that as parent flock age increases hatching time increases but in their experiment two strains represented as N and B were used, which are believed to be Nicholas and British United turkey strains. This agrees with the current finding in N strain in which hatching time increased with flock age between Y and M flocks. However, Christensen et al. (2001a) did not find significant difference between the interaction of genetic strain and flock age. The differences in hatching time observed with the N strain could be unique to this particular strain which also had higher G than H strain.

A previous study has shown that in turkeys, a special relationship exists between egg weight, G and incubation length (Christensen et al., 2006c). The differences observed in G between parent flock ages from N strain (Table 4.1), coupled with the higher G in N strain may have combined to cause significant differences in the interaction between N strain and its parent flock ages. With both G and incubation length appearing to increase in the interaction between N strain and its older parent flock ages, Christensen et al. (2006c) further stated that these could increase eggshell conductance constant and reduced embryonic heart weight and embryo survival. However, heart rate and survivability of poults were not measured in the current study. There were no significant effects of interaction of genetic strain and parent and flock age on poult length, shank length, and poult carcass analysis (Table 4.4).

2.3. Embryonic metabolism and respiratory quotient

2.3.1. Genetic strain

Daily embryonic O_2 consumption was not significantly different between genetic strains from 4 to 28 d of incubation (Figure 4.1). Apart from d 27 (1,071.33 ± 31.99 ml/d versus 1,042.03 ± 40.21 ml/d) and d 28 (1,197.59 ± 29.84 ml/d versus 1,099.64 ± 31.09 ml/d) where embryos from H strain had higher daily CO_2 production than N strain, the daily CO_2 production did not differ between strains (Figure 4.2). The results may imply that the embryos from H strain have the potential to produce more heat than N strain during the last 2 d of incubation. However, this did not follow as expected because daily EHP did not differ between genetic strains in all days of incubation (Table 4.7). The lower CO_2 production observed in embryos from N strain in the last 2 d may promote embryonic survival because these embryos would escape suffocation from the build-up of CO_2 in the egg. Although there are variations in CO_2 production between genetic strains, the differences are so small that they may have no biological significance for the embryos. This could have resulted in the lack of differences in EHP and EHP/g in the last 2 d of incubation. Total embryonic O_2 consumption and CO_2 production over the entire incubation period did not differ between genetic strains (Table 4.11).

The embryos from the H strain had higher daily RQ on 14, 19, and 22 to 24 d of incubation than N strain (Table 4.7). For the rest of the days of incubation RQ was not significantly different between strains. The RQ values reported were not a reflection of the daily differences in O_2 and CO_2 concentrations reported for

d 27 and 28 d of incubation. It is more likely that the daily numerical differences in O_2 and CO_2 concentrations which did not differ significantly prior to d 27 and 28 of incubation had more biological effects on embryos in terms of nutrient utilization than those statistically reported in 27 and 28 d of incubation. Nevertheless, the RQ data showed that embryos from N strain compared to H embryos were metabolizing more fats and proteins than carbohydrates (Table 4.7). This assertion is based on comparison of RQ values observed in this study to the theoretical RQ values of carbohydrates, proteins, and fats which are 1.0, 0.8 and 0.7 respectively (Krogh and Lindhard, 1920). From 25 d of incubation daily RQ values did not differ between strains. Reidy et al. (1998) reported that prior to the plateau stage of O_2 consumption turkey embryos from different genetic strains may metabolize yolk nutrients differently. This agrees with the current study, where RQ values were different between strains before 25 d of incubation.

2.3.2. Parent flock age

Additionally, daily embryonic O_2 consumption was higher in embryos from O flock compared to embryos from all other parent flock ages during 4, 8, 11 to 21, 25 and 27 d of incubation (Figure 4.1). Daily O_2 consumption was significantly different between embryos from Y, P and M flocks during all the days of incubation except 7, 9 to 17, 21, 26 and 27 d of incubation. Like the O_2 consumption, the CO_2 production was significantly higher in O flock than all other parent flock ages at 4, 5, 7, 8, 10 to 25 and 27 d of incubation. The M flock generally had higher daily CO_2 production than P and Y flock (Figure 4.2). Total

embryonic O_2 consumption over the entire incubation period was higher in the O and P flocks compared to the M and Y flocks (Table 4.11). The total CO_2 production in the entire incubation period increased with increasing flock age (Table 4.11).

The higher O_2 consumption and CO_2 production of the O flock could be due to the higher G observed earlier in this study. This higher G could be allowing more gas exchange between eggs from O flock and their environment. The higher O_2 and CO_2 concentration however could result in higher EHP (Kleiber, 1987) in embryos from O flock and expose them to the risk of overheating during the later periods of incubation. In chickens, research show that as parent flock age increases, the embryos tend to produce more heat (Tona et al., 2004; Hamidu et al., 2007) and this has been confirmed by anecdotal information from the poultry industry too. While Christensen et al., (1999, 2004) has reported that higher O_2 consumption around the plateau stage of O_2 consumption is important for hatching success; the current study did not measure hatchability or embryonic mortality and thus could not report that increase in O_2 consumption by embryos from O flock was an advantage over embryos from other flock ages. However in terms of hatch time, this result disagrees with the theory that increases in CO_2 production triggers early hatching process (Visschedijk, 1985). Although, embryos from the O flock produced the most CO_2, followed by embryos from M flock during the entire incubation period than embryos from Y and P flock ages, it took these embryos longer to hatch. The relatively higher O_2 consumption and CO_2 production observed in embryos from O and M flocks could imply a higher

metabolism of the fat portion of the yolk need to enhance growth. This is confirmed by the higher embryo weights recorded for O and M flocks in this study (Table 4.5).

Whereas total EHP was not significantly different between parent flock ages, EHP, the parameter used as an indication of embryonic metabolism (sum total of all metabolic activities including maintenance and growth) was significantly different between parent flock ages on a daily basis (Table 4.8). However, for 8 d (P=0.1298), 25 d (P=0.0688) and 28 d (P=0.2347) of incubation EHP did not differ between embryos from all parent flock ages. For the periods, 4, 6, 9, 11 to 24, 26 and 27 d of incubation embryos from the O flock produced more EHP than the Y flock. This agrees with earlier expectation that embryos from O flock would have higher EHP due to increased O_2 consumption and CO_2 production. In all animals, embryonic cell numbers increase by mitotic cell division to increase embryo size (Halevy, et al., 2006). In turkeys, because embryos from older parent flocks are normally bigger than embryos from younger parent flocks (Lewis and Morris, 1998) it can be assumed that they have more cells. Therefore, the increased embryonic EHP in older parent flock ages could be related to the number of embryonic cells present. The lack of variation in total EHP over the entire incubation period suggests that daily and not total EHP is more important in determining the effects of parent flock age on embryonic metabolism (Hamidu et al., 2007), and potentially in determining optimum incubation conditions.

On a daily basis, embryos from the Y flock had higher daily EHP/g embryo weight during 4 d (167 mW/g), 8 d (24.1 mW/g) and 13 d (8.37 mW/g) of incubation compared to all other flock ages ($P<0.0001$) (Table 4.9). On d 7, EHP/g of the Y flock embryos was higher than that of M and O flock embryos but not higher than P flock embryos. Whereas embryos from Y and O flocks had a higher EHP/g at 14 d of incubation than embryos from P and M flocks, the embryos from the O flock had higher EHP/g than the M flock at 19 d of incubation. The EHP/g from both M and O flocks did not differ from embryos of either the Y or P flock at 19 d of incubation. Between 19 and 23 d of incubation, the Y, P and O flock embryos had significantly higher EHP/g than the embryos from the M flock. From 23 to 28 d of incubation the M flock embryos consistently had lower EHP/g compared to embryos from all other flock ages.

The results from 4 to 13 d of incubation in embryos from the Y flock indicate that during the early period of incubation embryos from Y flock were metabolizing at a higher rate to gain similar body weight as those from other flock ages. Previous research shows that early on embryonic mortality in younger hens compared to older hens was significantly greater. The authors reported that this was true for mortality prior to blood formation and mortality following blood formation (Fairchild et al., 2002). Therefore, the higher early embryonic mortality may be related to the higher EHP/g embryo in embryos from the Y flock. Also EHP/g of body weight may be related to incubation temperatures of the Y flock. If the embryos from Y flock were incubating at lower than the normal temperature, this could expose them to hypothermia which limits their growth.

This agrees with well known evidence that lower incubation temperature slows down embryo development while higher temperature increases embryo body weight (Albokhadaim et al., 2010). The higher EHP/g of body weight could mean that the embryo were metabolizing at a higher rate compared to the embryos from older flock ages. This could be a form of physiological mechanism used by lower weight embryos to elevate their body temperature to increase their survival. We assume that increasing incubator temperature for embryos from Y flock may further hasten their metabolism and improve their development or survival. Even though the EHP was shown to be greater in the embryos from the O flock during the late period of incubation than embryos of all other flock ages, it appears that embryos from Y and P were metabolizing at an extra rate to gain heavier body weight in the later part of incubation compared to M and O flocks (except 26 d). These are similar to observations made in the embryos from the Y flock during the early period of incubation.

With the exception of 5, 6, 8 to 11, 15 to 17, 24, 26 and 28 d of incubation where daily average RQ values did not differ between flock ages (Table 4.10), daily RQ values were significantly different between flock ages. The RQ was significantly higher in embryos from O flock than those of the M flock at 4, 7, 12 to 14, 18, 20 to 23 and 25 d of incubation. The embryos from the O flock also had higher RQ at 12 to 14, 18, 19, 22, 23 and 25 d of incubation compared to embryos from the Y flock. However, the RQ values of embryos from the O flock were only higher at 12, 13, and 18 d of incubation than embryos from the P flock. In general RQ values were higher in O flock compared to Y flock. Earlier in this study, it

was assumed that, since the embryos from the O flock consumed more O_2 and produced more CO_2, they were metabolizing fats rather than carbohydrates and proteins. The higher RQ values recorded for embryos from the O flock now show that these embryos are not necessarily consuming more O_2 for metabolism. In relation to their bigger BW, these embryos were probably consuming less O_2 compared to embryos from other flock ages and producing more CO_2. It could also be that the embryos from the O flock were actively involved in metabolizing simple molecules such as carbohydrates in the yolk with little O_2 (Christensen et al., 1999). On the other hand, embryos from the O flock may not have been using more fat in the complex β-oxidation pathway which requires more O_2 in relation to their body size to produce energy. The reduced utilization of fats and proteins in the embryos from O flock could also be the reason why they had higher RYS (dry matter (DM)) after hatch. On the contrary, smaller RQ values observed in embryos from Y flock from 12 d of incubation onwards may indicate an intensive utilization of yolk fats to produce energy for growth. This could have accounted for the larger wet YFMB and smaller RYS (wet and DM) observed in poults hatched from Y flock.

In general RQ values decreased with days of incubation especially for embryos from Y and O parent flock ages. This shows that RQ values or nutrients used by embryos during incubation are not the same but change from more simple molecules such as carbohydrates to more complex molecules like proteins and fats by the time the embryos is closed to hatching. Proteins and fats however may contain many multiple bonds and therefore may require more oxygenation for

combustion and thus bringing down the RQ values. In general the RQ values appeared relatively higher during some days, especially from 12 d to 20 d of incubation in all flocks, which are periods embryos predominantly use fats in the yolk during metabolism. However, a previous study has shown that embryos from modern strains of turkey selected for growth had different energy metabolism that relied more on gluconeogenesis (Christensen et al., 2008). It is also know that in this type of metabolism fats are broken down to produce glucose and then utilized in the glycolysis pathway. This could be the reason why there were relatively higher RQ values than previously reported for chicken embryos (Hamidu et al., 2007). However, from 24 d onwards the higher RQ could be due to the normal shift to carbohydrates during pipping. Before the plateau phase of O_2 consumption, glycogen is formed and stored in the liver.

During the beginning of the plateau phase, this process ends and the glycogen is used during the plateau phase for pipping and hatching resulting in higher RQ values (Christensen et al., 1999). However, after the plateau phase all the carbohydrates are used up and the embryo again enters into fat metabolism resulting in smaller RQ (Dietz et al., 1998; O'Dea et al., 2004). This is true in the current study in all flocks at 27 and 28 d excluding P flocks at 27 d. In some embryos the carbohydrate reserve in the liver may be depleted during the plateau phase so that there is not enough energy during the pipping and the hatching processes causing the embryo to be exhausted after hatching (Christensen and Donaldson, 1992; Christensen et al., 2000, 2001b). This could be a cause for most late embryo mortality (Freeman, 1969). The higher RQ in embryo from the O

flock may have been because they were not using the yolk nutrients which are basically fats. This could also have resulted in the larger percentage of RYS observed in embryos from the O flock. Because RQ value increased in the O flock which was unexpected, it will be worthwhile to repeat this experiment with particular interest in the RQ values and carcass composition of poult to determine any correlation between RQ and YFBM and RYS.

2.3.3. Interaction of genetic strain and parent flock age

Interactively, there were no differences in total embryonic O_2 consumption, CO_2 production and EHP due to the interaction of genetic strain and parent flock age (Table 4.11). The average RQ values over the entire incubation period were significantly different due to the interaction of genetic strain and parent flock age (Table 4.11). The N by O embryos had the highest RQ (0.90 ± 0.02) and the N by M embryos had the lowest RQ (0.74 ± 0.02). The results may suggest that embryos from the same strain have the ability to utilize carbohydrates, proteins or lipids in the yolk at the same time. However, this is also dependent on parent flock age. Christensen et al. (1999) reported that at the plateau stage of O_2 consumption while turkey embryos from one strain utilized more fats than carbohydrates by consuming more O_2, the second strain used enough fats as carbohydrates. Currently, in incubation, nutrients can be *in ovo* injected into eggs before the embryo is hatched (Foye et al., 2006); RQ values may be an excellent indication of which nutrients are being metabolized by the embryo at this stage. During pipping and hatching, embryos resort to

carbohydrates metabolism as they need readily available energy (carbohydrates) rather than spending more energy to generate energy in fat metabolism (O'Dea et al., 2004). In some embryos the carbohydrate reserves may be depleted during the pipping and the hatching processes causing the embryo to be exhausted after hatching (Christensen and Donaldson, 1992; Christensen et al., 2000, 2001b). This could be a cause for most late embryo mortality (Freeman, 1969). In flocks such as the N by M interaction where RQ was low, the embryos could be provided with carbohydrate supplements to boast their energy level and eliminate possible late embryo mortality due to "anergia". Recent development of in ovo injections procedures could be useful in this regard.

2.4. Summary and conclusions

Some hatcheries are changing from the use of multi-stage incubation to the use of single-stage incubation. This is because differences between eggs from varying genetic strains and flock ages make it difficult to tailor incubation conditions to the need of all embryos, especially when eggs from different sources (strain and parent flock) are incubated together. In this chapter, embryonic metabolism was used as a sign of overheating in different turkey strains and flock ages. In a similar study we have conducted using modern broiler breeder eggs we showed that embryos from older parent flock ages produced more heat toward the end of the incubation period. This has also been confirmed by the results of our current research. Whereas in the broiler breeders difference were due to both strain and flock age, in the current study the impact of flock age was found to be

more influential than strain. In particular, embryos from older flock ages (55 and 60 week) had the potential to produce more heat than younger flocks (30 and 34 week). The differences between the two extreme flock ages (30 and 60 wk) were clear. The embryonic heat production presented between these two flock ages appears to indicate that turkey eggs from different flock ages should not be treated similarly during incubation. Though temperature can influence metabolism, it is not clear if incubating eggs from different parent flock ages under the same incubation temperature during the later part of incubation influenced embryonic metabolism. It is proposed that different incubation temperatures should be investigated for all flock ages to determine at what temperature embryos from each flock age has the desired heat production where overheating is reduced.

The higher EHP/g of embryo weight in the Y flock compared to other flock ages during early and mid incubation indicates that embryos from the Y flock are metabolizing at a higher rate in order to gain similar gram of BW as embryos from the other parent flock ages. The embryos from the Y flock may have been incubated at lower or higher than their required temperature. Thus, they could be trying to metabolize at a higher rate. However, it is not clear if the higher rate of metabolism may be the cause of increased first 7 d early embryonic mortality reported for embryos of younger parent flock ages (Fairchild et al., 2002). A developing avian embryo up to about 3 wk posthatch is a poikilotherm (Tazawa et al., 1988). In chickens, exposure of the poikilothemic embryo to a low incubation temperature slowed the development of the embryo, and in turn reduced metabolic rate and energy production. The embryonic growth and

maturation were also slowed down (Tazawa et al., 1988; Black and Burggren, 2004). Therefore, there is the need to decrease or raise the temperature of embryos from Y flock in future research to determine if any relationship exist between metabolic rate and decrease or increase in incubation temperature.

Because previous research has shown that early and late incubation embryonic mortality are higher in younger and older flocks respectively (Fairchild et al., 2002). Additional research is needed to investigate embryonic mortality between turkey parent flock ages and how this could be related to embryonic metabolism during early and late periods of incubation. If future research is able to establish any relationship between embryonic metabolism, incubation temperature and embryonic mortality, these results could be very useful for hatchery managers when deciding which eggs to incubate together for optimum hatchability. From the current results, eggs from different genetic strains (H and N) could likely be incubated together without any problem, since there are no differences in metabolism between the two strains but not for flock ages.

Table 4.1. The effects of 2 turkey genetic strains, 4 parent flock ages and their interaction on egg weight, eggshell thickness and eggshell conductance

Source	Initial egg weight[1] (g)	Egg moisture loss [2] (%)	Eggshell thickness[3] (mm)	Conductance [4] (g/d per mmHg)
Genetic strain				
Hybrid (H)	88.15 [b] (60)[5]	4.79 (59)	0.373 (59)	20.36 [b] (59)
Nicholas (N)	91.58 [a] (60)	5.08 (60)	0.365 (60)	22.29 [a] (60)
SEM	0.88	0.14	0.006	0.63
Flock age				
Young (Y – 30 wk)	75.09 [c] (30)	4.04 [c] (29)	0.360 [bc] (29)	17.20 [c] (29)
Peak (P – 34 wk)	88.30 [b] (30)	4.99 [b] (30)	0.379 [ab] (30)	19.24 [c] (30)
Mature (M – 55 wk)	97.16 [a] (30)	4.91 [b] (30)	0.340 [c] (30)	22.60 [b] (30)
Old (O – 60 wk)	98.91 [a] (30)	5.81 [a] (30)	0.397 [a] (30)	26.26 [a] (30)
SEM	1.24	0.20	0.009	0.88
Strain x Flock Age				
Hybrid x Young	76.35 [d] (15)	4.05 (14)	0.377 (14)	17.71 [bc] (14)
Hybrid x Peak	86.20 [c] (15)	4.66 (15)	0.381 (15)	17.53 [bc] (15)
Hybrid x Mature	93.29 [b] (15)	4.46 (15)	0.344 (15)	19.73 [b] (15)
Hybrid x Old	96.77 [ab] (15)	6.00 (15)	0.391 (15)	26.46 [a] (15)
Nicholas x Young	73.83 [d] (15)	4.02 (15)	0.343 (15)	16.70 [c] (15)
Nicholas x Peak	90.39 [bc] (15)	5.32 (15)	0.377 (15)	20.96 [b] (15)
Nicholas x Mature	101.04 [a] (15)	5.36 (15)	0.337 (15)	25.47 [a] (15)
Nicholas x Old	101.05 [a] (15)	5.62 (15)	0.403 (15)	26.05 [a] (15)
SEM	1.76	0.28	0.012	1.24

[a – d] Columnar means with different superscripts differ significantly ($P \leq 0.05$).

[1] Initial egg weight = egg weight at the time of collection.

[2] Egg moisture loss = ((final egg weight – initial egg weight)/initial egg weight) X 100.

[3] Eggshell thickness = width of the eggshell and membranes at the equator.

[4] Conductance = rate of moisture loss / saturated vapor pressure.

[5] (n) = number of experimental units; each experimental unit = each egg.

Table 4.2. The effects of 2 turkey genetic strains and 4 parent flock ages on egg components

Source	Wet yolk [1] (%)	Wet shell [2] (%)	Wet albumen [3] (%)	Dry yolk [4] (%)	Dry shell [5] (%)
Genetic strain					
Hybrid (H)	29.73 [a] (53) [6]	9.15 [a] (60)	61.09 [b] (53)	17.50 (53)	9.02 (60)
Nicholas (N)	28.41[b] (56)	8.77 [b] (60)	62.80 [a] (56)	16.65 (55)	8.68 (60)
SEM	0.39	0.09	0.41	0.39	0.08
Flock age					
Young (Y – 30 wk)	26.09 [b] (30)	9.22 [a] (30)	64.69 [a] (30)	14.38 [b] (30)	9.16 [a] (30)
Peak (P – 34 wk)	26.83 [b] (26)	9.53 [a] (30)	63.63 [a] (26)	14.47 [b] (25)	9.41 [a] (30)
Mature (M – 55 wk)	31.01 [a] (25)	8.67 [b] (30)	60.26 [b] (25)	19.74 [a] (25)	8.55 [b] (30)
Old (O – 60 wk)	32.33 [a] (28)	8.42 [b] (30)	59.21 [b] (28)	19.72 [a] (28)	8.27 [b] (30)
SEM	0.55	0.12	0.57	0.54	0.12
Strain x Flock Age					
Hybrid x Young	26.23 (15)	9.48 (15)	64.29 (15)	14.49 (15)	9.4138 (15)
Hybrid x Peak	27.68 (13)	9.69 (15)	62.62 (13)	14.84 (13)	9.4789 (15)
Hybrid x Mature	32.48 (11)	8.84 (15)	58.60 (11)	20.28 (11)	8.7161 (15)
Hybrid x Old	32.51 (14)	8.59 (15)	58.87 (14)	20.41 (14)	8.479 (15)
Nicholas x Young	25.95 (15)	8.97 (15)	65.09 (15)	14.26 (15)	8.9107 (15)
Nicholas x Peak	25.98 (13)	9.37 (15)	64.64 (13)	14.10 (12)	9.3418 (15)
Nicholas x Mature	29.54 (14)	8.49 (15)	61.93 (14)	19.20 (14)	8.3914 (15)
Nicholas x Old	32.15 (14)	8.26 (15)	59.55 (14)	19.03 (14)	8.0734 (15)
SEM	0.78	0.17	0.81	0.77	0.16

[a – b] Columnar means with different superscripts differ significantly ($P \leq 0.05$).

[1] Wet yolk (%) = (wet yolk weight/initial egg weight) x 100.

[2] Wet shell (%) = (wet shell weight/initial egg weight) x 100.

[3] Albumen (%) = (final egg weight - (wet yolk + wet shell)) x 100.

[4] Dry yolk (%) = (dry yolk weight/initial egg weight) x 100.

[5] Dry shell (%) = (dry shell weight/initial egg weight) x 100.

[6] (n) = number of experimental units; each experimental unit = each yolk or shell.

Table 4.3. The effects of 2 turkey genetic strains, 4 parent flock ages and their interaction on egg and poult characteristics

Source	Egg set weight [1] (g)	Transfer weight [2] (g)	Pipping time [3] (h)	Hatching time [4] (h)	Poult weight [5] (g)
Genetic strain					
Hybrid (H)	87.89 (44) [6]	78.23 (44)	629.14 (40)	652.97 (32)	64.52 (32)
Nicholas (N)	89.10 (44)	77.76 (44)	629.07 (38)	652.4 (34)	64.22 (34)
SEM	0.79	0.78	1.44	1.49	0.79
Flock age					
Young (Y – 30 wk)	71.89 [d] (22)	63.37 [c] (22)	622.10 [b] (20)	645.50 [b] (16)	51.34 [c] (17)
Peak (P – 34 wk)	89.05 [c] (22)	78.14 [b] (22)	624.60 [b] (18)	649.07 [b] (15)	64.15 [b] (18)
Mature (M – 55 wk)	94.99 [b] (22)	85.27 [a] (22)	633.57 [a] (19)	657.75 [a] (18)	71.10 [a] (15)
Old (O – 60 wk)	98.12 [a] (22)	85.22 [a] (22)	636.17 [a] (18)	658.50 [a] (17)	70.89 [a] (16)
SEM	1.11	1.10	2.04	2.12	1.11
Strain x Flock Age					
Hybrid x Young	76.44 [c] (11)	67.77 [d] (11)	622.40 (10)	649.25 [bc] (8)	54.22 (9)
Hybrid x Peak	86.92 [b] (11)	77.07 [c] (11)	623.70 (10)	648.64 [bc] (7)	63.26 (10)
Hybrid x Mature	90.96 [b] (11)	83.04 [ab] (11)	630.30 (10)	652.50 [bc] (10)	70.03 (7)
Hybrid x Old	97.25 [a] (11)	85.08 [a] (11)	640.17 (9)	661.50 [ab] (9)	70.56 (8)
Nicholas x Young	67.35 [d] (11)	59.01 [e] (11)	621.80 (10)	641.75 [c] (8)	48.46 (8)
Nicholas x Peak	91.18 [b] (11)	79.21 [bc] (11)	625.50 (8)	649.50 [bc] (8)	65.04 (8)
Nicholas x Mature	99.03 [a] (11)	87.50 [a] (11)	636.83 (9)	663.00 [a] (8)	72.17 (8)
Nicholas x Old	98.99 [a] (11)	85.35 [a] (11)	632.17 (9)	655.50 [b] (8)	71.22 (8)
SEM	1.57	1.55	2.69	2.99	1.56

[a-d] Columnar means with different superscripts differ significantly ($P \leq 0.05$).

[1] Initial weight of egg after collection and before it was placed in incubator.

[2] Egg weight at 25 d of incubation.

3 Time taken from setting eggs in incubator to external pipping.

[4] Time taken from setting eggs in incubator to when poult hatched and down feathers dry.

[5] Weight of poult after hatching.

[6] Number of experimental units; each experimental unit = each egg or poult.

Table 4.4. The effects of 2 turkey genetic strains and 4 parent flock ages on poult and poult carcass characteristics

Source	Poult length [1] (mm)	Shank length [2] (mm)	Wet YFBM [3] (%)	Wet RYS [4] (%)	Dry YFBM [5] (%)	Dry RYS [6] (%)
Genetic strain						
Hybrid (H)	212.21 (32) [7]	31.64 (32)	83.76 (32)	15.61(32)	17.06 (32)	8.65 (32)
Nicholas (N)	210.78 (34)	31.41 (34)	83.04 (34)	16.44 (34)	17.67 (34)	8.89 (34)
SEM	1.07	0.28	0.47	0.42	0.32	0.24
Flock age						
Young (Y – 30 wk)	202.90[c] (17)	32.25 [a] (16)	87.42 [a] (17)	12.00 [c] (17)	17.01 [a] (17)	5.70 [c] (17)
Peak (P – 34 wk)	210.78[b] (18)	31.47 [a] (15)	85.93 [a] (18)	13.68 [b] (18)	17.96 [a] (18)	7.04 [b] (18)
Mature (M – 55 wk)	216.20[a] (15)	32.05 [a] (18)	79.93 [b] (15)	19.57 [a] (15)	15.65 [b] (15)	11.33 [a] (15)
Old (O – 60 wk)	216.13[a] (16)	30.32 [b] (17)	80.33 [b] (16)	18.84 [a] (16)	17.14 [a] (16)	11.02 [a] (16)
SEM	1.52	0.39	0.66	0.58	0.42	0.34
Strain x Flock Age						
Hybrid x Young	206.67 (9)	30.89 (9)	87.88 (9)	11.28 (9)	17.41 (9)	5.35 (9)
Hybrid x Peak	210.30 (10)	31.60 (10)	85.82 (10)	13.62 (10)	16.94 (10)	6.96 (10)
Hybrid x Mature	216.14 (7)	31.57 (7)	80.20 (7)	19.28 (7)	15.62 (7)	11.54 (7)
Hybrid x Old	215.75(8)	32.50 (8)	81.15 (8)	18.26 (8)	18.26 (8)	10.74 (8)
Nicholas x Young	199.12 (8)	29.75 (8)	86.96 (8)	12.72(8)	16.61 (8)	6.04 (8)
Nicholas x Peak	211.25 (8)	32.50 (8)	86.06 (8)	13.74 (8)	18.97 (8)	7.12 (8)
Nicholas x Mature	216.25 (8)	31.39 (8)	79.66 (8)	19.86 (8)	15.69 (8)	11.12 (8)
Nicholas x Old	216.50 (8)	32.00 (8)	79.52 (8)	19.42 (8)	19.42 (8)	11.29 (8)
SEM	2.14	0.55	0.93	0.83	0.62	0.48

[a-c] Columnar means with different superscripts differ significantly ($P \leq 0.05$).

[1] The distance between poult beak and tip of the longest toe.

[2] The distance along the metatarsus.

[3] (Wet poult carcass weight/poult weight) x100.

[4] Wet yolk sac weight/poult weight) x 100.

[5] (Dry poult carcass weight/poult weight) x100.

[6] (Dry yolk sac weight/poult weight) x 100.

[7] Number of experimental units; each experimental unit = each poult or yolk sac.

Table 4.5. The effects of 2 turkey parent flock ages on daily embryo weight during each of 4 to 28 d of incubation

Day of incubation	Embryo weight (g)			
	Young (Y – 30 wk)	Peak (P – 34 wk)	Mature (M – 55 wk)	Old (O – 60 wk)
4	0.006 ± 0.003 [c] (16) [2]	0.010 ± 0.003 [c] (14)	0.031 ± 0.002 [a] (18)	0.022 ± 0.002 [b] (21)
5	0.021 ± 0.011 [b] (16)	0.031 ± 0.012 [b] (13)	0.056 ± 0.009 [ab] (24)	0.074 ± 0.009 [a] (24)
6	0.065 ± 0.009 [b] (27)	0.081 ± 0.012 [b] (17)	0.144 ± 0.010 [a] (25)	0.153 ± 0.010 [a] (23)
7	0.187 ± 0.016 [b] (20)	0.211 ± 0.018 [b] (16)	0.314 ± 0.015 [a] (23)	0.330 ± 0.015 [a] (23)
8	0.353 ± 0.021 [c] (22)	0.440 ± 0.024 [b] (17)	0.607 ± 0.021 [a] (23)	0.602 ± 0.020 [a] (25)
9	0.629 ± 0.032 [c] (21)	0.817 ± 0.036 [b] (17)	1.080 ± 0.030 [a] (24)	1.045 ± 0.028 [a] (27)
10	1.100 ± 0.042 [c] (23)	1.245 ± 0.050 [b] (16)	1.519 ± 0.043 [a] (22)	1.561 ± 0.043 [a] (22)
11	1.553 ± 0.046 [d] (20)	1.915 ± 0.041 [c] (25)	2.252 ± 0.043 [a] (23)	2.123 ± 0.041 [b] (25)
12	2.485 ± 0.061 [b] (21)	2.555 ± 0.057 [b] (24)	2.825 ± 0.056 [a] (25)	2.848 ± 0.059 [a] (22)
13	3.007 ± 0.114 [c] (15)	3.484 ± 0.090 [b] (24)	3.911 ± 0.098 [a] (20)	3.638 ± 0.090 [ab] (24)
14	4.037 ± 0.136 [b] (20)	4.760 ± 0.117 [a] (27)	5.055 ± 0.127 [a] (23)	4.969 ± 0.117 [a] (27)
15	5.610 ± 0.121 [b] (23)	5.840 ± 0.124 [b] (22)	6.612 ± 0.124 [a] (22)	6.849 ± 0.130 [a] (20)
16	6.702 ± 0.231 [b] (18)	8.123 ± 0.192 [a] (26)	8.445 ± 0.196 [a] (25)	8.795 ± 0.209 [a] (22)
17	10.044 ± 0.278 [c] (15)	10.625 ± 0.225 [bc] (23)	11.283 ± 0.235 [b] (21)	12.297 ± 0.220 [a] (24)
18	12.788 ± 0.344 [b] (13)	15.483 ± 0.265 [a] (22)	15.466 ± 0.248 [a] (25)	15.438 ± 0.253 [a] (24)
19	17.066 ± 0.496 [c] (11)	18.725 ± 0.343 [b] (23)	20.704 ± 0.343 [b] (23)	19.286 ± 0.350 [a] (22)
20	20.137 ± 1.665 [b] (22)	23.286 ± 1.704 [ab] (21)	25.260 ± 1.665 [ab] (22)	26.813 ± 1.628 [a] (23)
21	23.850 ± 0.453 [d] (20)	26.932 ± 0.441 [c] (21)	30.330 ± 0.413 [a] (24)	28.907 ± 0.405 [b] (25)
22	26.954 ± 0.881 [b] (18)	30.607 ± 0.747 [a] (25)	33.152 ± 0.815 [a] (21)	31.913 ± 0.797 [a] (22)
23	31.114 ± 0.632 [c] (24)	36.279 ± 0.607 [b] (26)	40.153 ± 0.632 [a] (24)	38.928 ± 0.660 [a] (22)
24	34.860 ± 0.781 [c] (22)	38.693 ± 0.748 [b] (24)	43.363 ± 0.800 [a] (21)	43.576 ± 0.764 [a] (23)
25	38.758 ± 0.958 [c] (23)	44.115 ± 0.938 [b] (24)	46.711 ± 0.958 [ab] (23)	49.073 ± 0.919 [a] (25)
26	45.725 ± 1.515 [d] (18)	53.647 ± 1.371 [c] (22)	65.234 ± 1.340 [b] (23)	60.349 ± 1.340 [a] (23)
27	50.736 ± 1.331 [c] (22)	58.439 ± 1.472 [b] (18)	68.463 ± 1.472 [a] (20)	67.876 ± 1.395 [a] (18)
28	51.043 ± 1.352 [c] (17)	61.065 ± 1.188 [b] (22)	66.124 ± 1.216 [a] (21)	68.727 ± 1.093 [a] (26)

[a-d] Means within the same row with different superscripts differ significantly ($P \leq 0.05$).

[1] Daily weight of embryos during each d of incubation from 4 to 28 d of incubation.

[2] Number experimental units; each experimental unit = each embryo.

Table 4.6. The effects of genetic strain on daily embryonic O_2 consumption and CO_2 production over 28 days of incubation

Day of incubation	O_2 consumption[1] (ml/d)		CO_2 production[2] (ml/d)	
	Hybrid	Nicholas	Hybrid	Nicholas
4	5.47 ± 0.23	5.33 ± 0.23	5.50 ± 0.23	5.36 ± 0.23
5	10.99 ± 0.72	8.88 ± 0.69	9.95 ± 0.36	9.80 ± 0.36
6	13.15 ± 0.68	12.13 ± 0.70	14.13 ± 0.50	13.10 ± 0.48
7	20.52 ± 3.66	24.93 ± 3.71	19.77 ± 0.73	18.83 ± 0.74
8	34.65 ± 2.02	35.86 ± 1.93	25.60 ± 0.95	26.70 ± 0.93
9	38.40 ± 1.71	39.06 ± 1.71	35.10 ± 4.49	40.83 ± 4.49
10	50.05 ± 3.78	55.57 ± 3.88	45.24 ± 1.24	44.94 ± 1.26
11	68.46 ± 3.17	65.75 ± 3.09	61.02 ± 2.63	61.27 ± 2.63
12	91.03 ± 2.40	90.62 ± 2.37	79.19 ± 1.78	79.64 ± 1.76
13	112.36 ± 2.03	111.71 ± 2.04	98.51 ± 1.74	98.90 ± 1.74
14	144.76 ± 3.56	147.88 ± 3.55	131.08 ± 4.41	136.43 ± 4.35
15	201.12 ± 5.46	198.27 ± 5.40	172.58 ± 5.28	176.46 ± 5.28
16	269.33 ± 6.14	262.80 ± 5.91	243.07 ± 5.25	247.20 ± 5.06
17	352.18 ± 8.55	343.26 ± 8.36	315.16 ± 7.21	312.54 ± 7.17
18	444.96 ± 8.67	440.12 ± 8.57	402.57 ± 8.05	399.26 ± 7.95
19	537.68 ± 8.65	536.17 ± 8.89	485.68 ± 7.86	477.99 ± 7.88
20	614.17 ± 9.37	604.39 ± 9.26	557.89 ± 8.66	550.16 ± 8.56
21	660.81 ± 11.48	654.79 ± 11.81	608.81 ± 10.76	612.79 ± 10.94
22	701.90 ± 13.48	709.71 ± 13.29	628.32 ± 12.36	622.37 ± 12.22
23	729.86 ± 13.24	736.56 ± 13.09	646.28 ± 12.45	653.91 ± 12.16
24	733.65 ± 13.93	743.15 ± 13.74	822.66 ± 17.20	502.88 ± 17.61
25	751.35 ± 11.84	744.44 ± 12.33	663.28 ± 12.20	655.83 ± 12.37
26	850.50 ± 37.13	848.93 ± 38.24	741.94 ± 35.82	751.77 ± 36.89
27	1207.04 ± 39.89	1177.90 ± 46.36	1071.33 ± 31.99 [a]	1042.03 ± 40.21 [b]
28	1301.82 ± 64.60	1353.87 ± 76.76	1197.59 ± 29.84 [a]	1099.64 ± 31.09 [b]

[a-b] Means within each d with different superscripts differ significantly ($P \leq 0.05$).

[1] Daily average O2 consumption from embryos within each strain.

[2] Daily average CO_2 production form embryos within each strain.

Table 4.7. The effects of 2 turkey genetic strains on daily embryonic heat production and daily respiratory quotient over 28 d of incubation

Day of incubation	Heat production [1] (mW)		Respiratory quotient [2]	
	Hybrid (H)	Nicholas (N)	Hybrid (H)	Nicholas (N)
4	1.33 ± 0.06	1.31 ± 0.05	0.98 ± 0.02	0.93 ± 0.02
5	2.61 ± 0.14	2.25 ± 0.13	0.93 ± 0.05	0.88 ± 0.05
6	3.21 ± 0.13	2.96 ± 0.14	0.98 ± 0.03	0.92 ± 0.04
7	4.63 ± 0.20	5.00 ± 0.20	0.92 ± 0.04	0.91 ± 0.04
8	6.96 ± 0.50	7.43 ± 0.50	0.82 ± 0.14	0.94 ± 0.14
9	8.61 ± 0.44	9.75 ± 0.44	0.85 ± 0.03	0.79 ± 0.03
10	11.47 ± 0.71	12.82 ± 0.73	0.83 ± 0.03	0.79 ± 0.03
11	15.45 ± 0.65	15.54 ± 0.65	0.80 ± 0.03	0.81 ± 0.03
12	20.37 ± 0.53	21.60 ± 0.53	0.80 ± 0.01	0.79 ± 0.02
13	25.81 ± 0.47	26.11 ± 0.47	0.79 ± 0.01	0.78 ± 0.01
14	33.46 ± 0.77	34.83 ± 0.78	0.84 ± 0.01 [a]	0.79 ± 0.02 [b]
15	46.02 ± 1.17	46.48 ± 1.16	0.77 ± 0.02	0.77 ± 0.02
16	61.10 ± 1.43	62.35 ± 1.38	0.81 ± 0.04	0.85 ± 0.03
17	81.81 ± 1.94	80.76 ± 1.93	0.80 ± 0.02	0.80 ± 0.01
18	102.99 ± 2.07	104.45 ± 2.05	0.84 ± 0.01	0.81 ± 0.01
19	125.01 ± 2.18	126.42 ± 2.18	0.84 ± 0.01 [a]	0.80 ± 0.01 [b]
20	143.90 ± 2.28	143.04 ± 2.25	0.83 ± 0.01	0.81 ± 0.01
21	153.30 ± 2.82	154.12 ± 2.87	0.80 ± 0.01	0.78 ± 0.02
22	161.19 ± 3.18	167.70 ± 3.10	0.81 ± 0.01 [a]	0.75 ± 0.02 [b]
23	167.42 ± 3.17	174.33 ± 3.09	0.81 ± 0.01 [a]	0.76 ± 0.01 [b]
24	168.32 ± 3.56	176.36 ± 3.47	0.83 ± 0.02 [a]	0.77 ± 0.02 [b]
25	170.70 ± 3.21	175.46 ± 3.30	0.80 ± 0.01	0.77 ± 0.01
26	207.58 ± 7.31	189.66 ± 7.52	0.83 ± 0.04	0.82 ± 0.04
27	269.68 ± 9.26	275.59 ± 8.83	0.74 ± 0.02	0.71 ± 0.02
28	295.95 ± 14.56	338.83 ± 16.41	0.72 ± 0.02	0.70 ± 0.03

[a-b] Means within each d with different superscripts differ significantly ($P \leq 0.05$).

[1] Daily average heat production from embryos within each strain.

[2] Daily CO_2 production /daily O_2 consumption.

Table 4.8. The effects of 4 turkey parent flock ages on daily embryonic heat production over 28 d of incubation

Day of incubation	Heat production [1] (mW)			
	Young (Y – 30 wk)	Peak (P – 34 wk)	Mature (M – 55 wk)	Old (O – 60 wk)
4	1.00 ± 0.07 [b]	0.93 ± 0.07 [b]	1.60 ± 0.07 [a]	1.75 ± 0.08 [a]
5	2.78 ± 0.20 [a]	1.63 ± 0.19 [b]	2.63 ± 0.19 [a]	2.66 ± 0.19 [a]
6	2.60 ± 0.20 [b]	2.43 ± 0.18 [b]	3.66 ± 0.20 [a]	3.69 ± 0.20 [a]
7	4.29 ± 0.30	4.82 ± 0.29	4.86 ± 0.27	5.29 ± 0.27
8	8.51 ± 0.73	6.05 ± 0.78	6.81 ± 0.66	7.42 ± 0.66
9	7.42 ± 0.63 [b]	9.74 ± 0.66 [a]	9.80 ± 0.61 [a]	9.75 ± 0.61 [a]
10	10.99 ± 1.04	12.04 ± 1.01	12.49 ± 0.97	13.05 ± 1.04
11	13.40 ± 1.07 [b]	14.66 ± 0.86 [ab]	16.41 ± 0.89 [ab]	17.51 ± 0.84 [a]
12	18.95 ± 0.78 [c]	19.98 ± 0.76 [bc]	21.63 ± 0.72 [ab]	23.36 ± 0.72 [a]
13	25.17 ± 0.67 [b]	24.36 ± 0.67 [b]	26.29 ± 0.64 [ab]	28.02 ± 0.64 [a]
14	32.69 ± 1.13 [b]	31.52 ± 1.10 [b]	34.68 ± 1.05 [ab]	37.69 ± 1.11 [a]
15	40.52 ± 1.67 [c]	44.76 ± 1.67 [bc]	48.46 ± 1.60 [ab]	51.27 ± 1.64 [a]
16	51.55 ± 1.99 [c]	60.53 ± 2.05 [b]	65.85 ± 1.95 [a]	70.76 ± 1.95 [a]
17	72.71 ± 2.94 [b]	76.13 ± 2.71 [b]	84.43 ± 2.65 [a]	91.86 ± 2.65 [a]
18	90.99 ± 2.97 [c]	103.31 ± 2.97 [b]	106.45 ± 2.83 [ab]	114.11 ± 2.90 [a]
19	111.26 ± 3.20 [b]	126.74 ± 3.11 [a]	128.47 ± 2.97 [a]	136.39 ± 3.04 [a]
20	128.74 ± 3.26 [c]	146.13 ± 3.26 [b]	143.62 ± 3.11 [b]	155.37 ± 3.19 [a]
21	141.08 ± 4.15 [b]	154.71 ± 4.15 [b]	149.23 ± 3.85 [b]	169.81 ± 3.95 [a]
22	148.03 ± 4.61 [b]	174.48 ± 4.48 [a]	162.82 ± 4.27 [a]	172.45 ± 4.38 [a]
23	152.59 ± 4.48 [c]	185.26 ± 4.48 [a]	166.72 ± 4.37 [b]	178.91 ± 4.37 [ab]
24	154.31 ± 5.02 [c]	188.21 ± 5.15 [a]	167.19 ± 4.78 [bc]	179.63 ± 4.90 [ab]
25	166.30 ± 4.60	180.42 ± 4.60	167.40 ± 4.62	178.21 ± 4.60
26	168.86 ± 10.33 [b]	198.45 ± 10.06 [ab]	197.20 ± 10.92 [ab]	229.97 ± 10.60 [a]
27	230.55 ± 10.19 [c]	264.77 ± 10.59 [bc]	288.42 ± 15.19 [ab]	306.79 ± 14.41 [a]
28	285.84 ± 25.00 [b]	352.51 ± 22.82 [a]	327.30 ± 23.57 [b]	303.91 ± 14.91 [b]

[a-c] Means within each d with different superscripts differ significantly ($P \leq 0.05$).

[1] Daily average heat production from embryos within each flock age

Table 4.9. The effects of 4 turkey parent flock ages on daily embryonic heat production per gram wet embryo weight over 28 d of incubation

Day of incubation	Heat production per gram of wet embryo weight [1] (mW/g)			
	Young (Y – 30 wk)	Peak (P – 34 wk)	Mature (M – 55 wk)	Old (O – 60 wk)
4	166.93 ± 7.01 [a]	93.12 ± 7.36 [b]	51.50 ± 7.55 [c]	79.56 ± 8.23 [b]
5	131.08 ± 9.02 [a]	52.68 ± 8.77 [b]	47.30 ± 8.53 [b]	35.63 ± 8.32 [b]
6	40.05 ± 2.66 [a]	30.05 ± 2.45 [b]	25.37 ± 2.66 [b]	23.81 ± 2.66 [b]
7	22.86 ± 1.16 [a]	22.87 ± 1.13 [a]	15.62 ± 1.07 [b]	16.02 ± 1.07 [b]
8	24.11 ± 1.81 [a]	13.75 ± 1.92 [b]	11.21 ± 1.64 [b]	12.33 ± 1.64 [b]
9	11.77 ± 0.81	11.98 ± 0.85	9.07 ± 0.79	9.33 ± 0.79
10	9.98 ± 0.83	9.67 ± 0.81	8.22 ± 0.77	8.37 ± 0.83
11	8.56 ± 0.53	7.66 ± 0.43	7.25 ± 0.44	8.25 ± 0.41
12	7.63 ± 0.33	7.82 ± 0.32	7.66 ± 0.30	8.20 ± 0.30
13	8.37 ± 0.21 [a]	6.99 ± 0.21 [c]	6.72 ± 0.20 [c]	7.70 ± 0.20 [b]
14	8.09 ± 0.24 [a]	6.62 ± 0.24 [b]	6.86 ± 0.22 [b]	7.58 ± 0.24 [a]
15	7.22 ± 0.28	7.66 ± 0.28	7.33 ± 0.27	7.47 ± 0.27
16	7.69 ± 0.27	7.43 ± 0.28	7.84 ± 0.26	8.04 ± 0.26
17	7.25 ± 0.29	7.17 ± 0.27	7.52 ± 0.26	7.46 ± 0.26
18	7.12 ± 0.21	6.67 ± 0.21	6.88 ± 0.20	7.39 ± 0.20
19	6.53 ± 0.18 [ab]	6.77 ± 0.18 [ab]	6.21 ± 0.17 [b]	7.07 ± 0.17 [a]
20	6.39 ± 0.15 [a]	6.28 ± 0.15 [a]	5.69 ± 0.14 [b]	5.79 ± 0.14 [b]
21	5.94 ± 0.17 [a]	5.75 ± 0.17 [a]	4.92 ± 0.16 [b]	5.87 ± 0.16 [a]
22	5.48 ± 0.16 [a]	5.70 ± 0.16 [a]	4.91 ± 0.15 [b]	5.41 ± 0.15 [a]
23	4.90 ± 0.14 [ab]	5.11 ± 0.14 [a]	4.17 ± 0.13 [c]	4.60 ± 0.13 [b]
24	4.43 ± 0.13 [b]	4.87 ± 0.14 [a]	3.86 ± 0.13 [c]	4.12 ± 0.13 [bc]
25	4.29 ± 0.12 [a]	4.09 ± 0.12 [a]	3.54 ± 0.12 [b]	3.63 ± 0.12 [b]
26	3.71 ± 0.18 [a]	3.70 ± 0.17 [a]	3.03 ± 0.19 [b]	3.81 ± 0.18 [a]
27	4.54 ± 0.19	4.47 ± 0.19	4.21 ± 0.28	4.52 ± 0.26
28	5.29 ± 0.16 [b]	6.20 ± 0.28 [a]	4.63 ± 0.31 [bc]	4.39 ± 0.22 [c]

[a-c] Means within each day with different superscripts differ significantly ($P \leq 0.05$).

[1]Daily heat production /daily wet embryo weight.

Table 4.10. The effects of 4 turkey parent flock ages on daily respiratory quotient over 28 d of incubation

Day of incubation	Respiratory quotient [1]			
	Young (Y – 30 wk)	Peak (P – 34 wk)	Mature (M – 55 wk)	Old (O – 60 wk)
4	1.02 ± 0.03 [a]	1.01 ± 0.03 [a]	0.85 ± 0.03 [b]	0.95 ± 0.03 [a]
5	0.83 ± 0.06	1.01 ± 0.09	0.94 ± 0.06	0.85 ± 0.07
6	0.97 ± 0.05	0.88 ± 0.04	0.97 ± 0.05	0.97 ± 0.05
7	0.89 ± 0.05 [ab]	0.99 ± 0.05 [a]	0.78 ± 0.05 [b]	1.02 ± 0.06 [a]
8	0.67 ± 0.20	0.84 ± 0.20	0.83 ± 0.18	1.19 ± 0.19
9	0.81 ± 0.05	0.82 ± 0.04	0.78 ± 0.04	0.88 ± 0.05
10	0.74 ± 0.05	0.86 ± 0.04	0.78 ± 0.04	0.86 ± 0.05
11	0.75 ± 0.04	0.86 ± 0.04	0.74 ± 0.05	0.87 ± 0.05
12	0.78 ± 0.03 [b]	0.73 ± 0.02 [b]	0.75 ± 0.03 [b]	0.92 ± 0.03 [a]
13	0.72 ± 0.02 [c]	0.79 ± 0.02 [b]	0.73 ± 0.02 [c]	0.89 ± 0.02 [a]
14	0.73 ± 0.02 [b]	0.86 ± 0.02 [a]	0.75 ± 0.03 [b]	0.91 ± 0.03 [a]
15	0.75 ± 0.03	0.75 ± 0.03	0.75 ± 0.02	0.83 ± 0.03
16	0.92 ± 0.05	0.80 ± 0.05	0.77 ± 0.05	0.83 ± 0.05
17	0.79 ± 0.03	0.81 ± 0.02	0.80 ± 0.02	0.82 ± 0.02
18	0.80 ± 0.02 [b]	0.80 ± 0.02 [b]	0.78 ± 0.02 [b]	0.92 ± 0.02 [a]
19	0.78 ± 0.02 [b]	0.81 ± 0.02 [ab]	0.84 ± 0.02 [a]	0.86 ± 0.02 [a]
20	0.81 ± 0.02 [ab]	0.82 ± 0.02 [ab]	0.79 ± 0.02 [b]	0.87 ± 0.02 [a]
21	0.75 ± 0.03 [b]	0.97 ± 0.03 [a]	0.63 ± 0.02 [c]	0.82 ± 0.03 [b]
22	0.75 ± 0.02 [b]	0.80 ± 0.02 [ab]	0.76 ± 0.02 [b]	0.83 ± 0.02 [a]
23	0.77 ± 0.02 [b]	0.79 ± 0.02 [ab]	0.76 ± 0.02 [b]	0.84 ± 0.02 [a]
24	0.77 ± 0.02	0.81 ± 0.02	0.79 ± 0.02	0.82 ± 0.03
25	0.76 ± 0.01 [b]	0.82 ± 0.01 [a]	0.74 ± 0.01 [b]	0.84 ± 0.01 [a]
26	0.76 ± 0.05	0.83 ± 0.05	0.85 ± 0.06	0.86 ± 0.05
27	0.75 ± 0.02 [a]	0.80 ± 0.03 [a]	0.64 ± 0.04 [b]	0.72 ± 0.03 [ab]
28	0.76 ± 0.03	0.68 ± 0.03	0.63 ± 0.05	0.76 ± 0.03

[a-c] Means within each day with different superscripts differ significantly ($P \leq 0.05$).

[1] Daily CO_2 production /daily O_2 consumption.

Table 4.11. The effects of 2 turkey genetic strains, 4 parent flock ages and their interaction on average gas exchange, respiratory quotient and heat production over 28 d of incubation

Source	Total O_2 consumption [1] (ml/d)	Total CO_2 production [2] (ml/d)	Average respiratory quotient [3] (RQ)	Total heat production [4] (mW)
Genetic strain				
Hybrid (H)	8976.80 (1076)[5]	7808.25 (1101)	0.85 (1012)	2104.99 (1074)
Nicholas (N)	8880.29 (1087)	7717.59 (1111)	0.83 (946)	2012.85 (1077)
SEM	297.33	256.84	0.01	81.98
Flock age				
Young (Y – 30 wk)	8,316.42 [b] (517)	6,029.82 [c] (554)	0.82 [b] (508)	1865.50 (520)
Peak (P – 34 wk)	9,570.20 [a] (525)	7,440.40 [b] (537)	0.86 [a] (513)	2240.60 (529)
Mature (M – 55 wk)	7,860.64 [b] (566)	7,853.18 [b] (568)	0.79 [b] (501)	2183.48 (555)
Old (O – 60 wk)	9,966.90 [a] (555)	9,728.28 [a] (553)	0.88 [a] (436)	1892.56 (547)
SEM	420.44	363.21	0.02	106.7
Strain * Flock Age				
Hybrid * Young	8,818.11 (262)	6,462.59 (279)	0.82 [c] (258)	2010.87 (264)
Hybrid * Peak	9,279.10 (259)	7,251.86 (266)	0.86 [bc] (256)	2123.38 (261)
Hybrid * Mature	7,977.23 (283)	7,953.99 (285)	0.84 [bc] (275)	1865.50 (280)
Hybrid * Old	9,832.74 (272)	9,564.56 (271)	0.87 [b] (223)	2239.23 (269)
Nicholas * Young	7,814.73 (255)	5,597.05 (275)	0.82 [c] (250)	1774.24 (256)
Nicholas * Peak	9,861.35 (266)	7,628.95 (271)	0.85 [bc] (257)	2243.58 (268)
Nicholas * Mature	7,744.04 (283)	7,752.37 (283)	0.74 [d] (226)	2160.19 (275)
Nicholas * Old	10,101.0 (283)	9,892.00 (282)	0.90 [a] (213)	2241.96 (278)
SEM	594.56	513.64	0.02	137.13

[a-d] Means within the same column with different superscripts differ significantly ($P \leq 0.05$).

[1] Sum of all average daily O_2 consumption for all 25 d of incubation.

[2] Sum of all average daily CO_2 production for all 25 d of incubation.

[3] Sum of all average daily respiratory quotients /25 d of incubation.

[4] Sum of average daily embryonic heat production over 25 d of incubation.

[5] Number of experimental units; each experimental unit = each air sample.

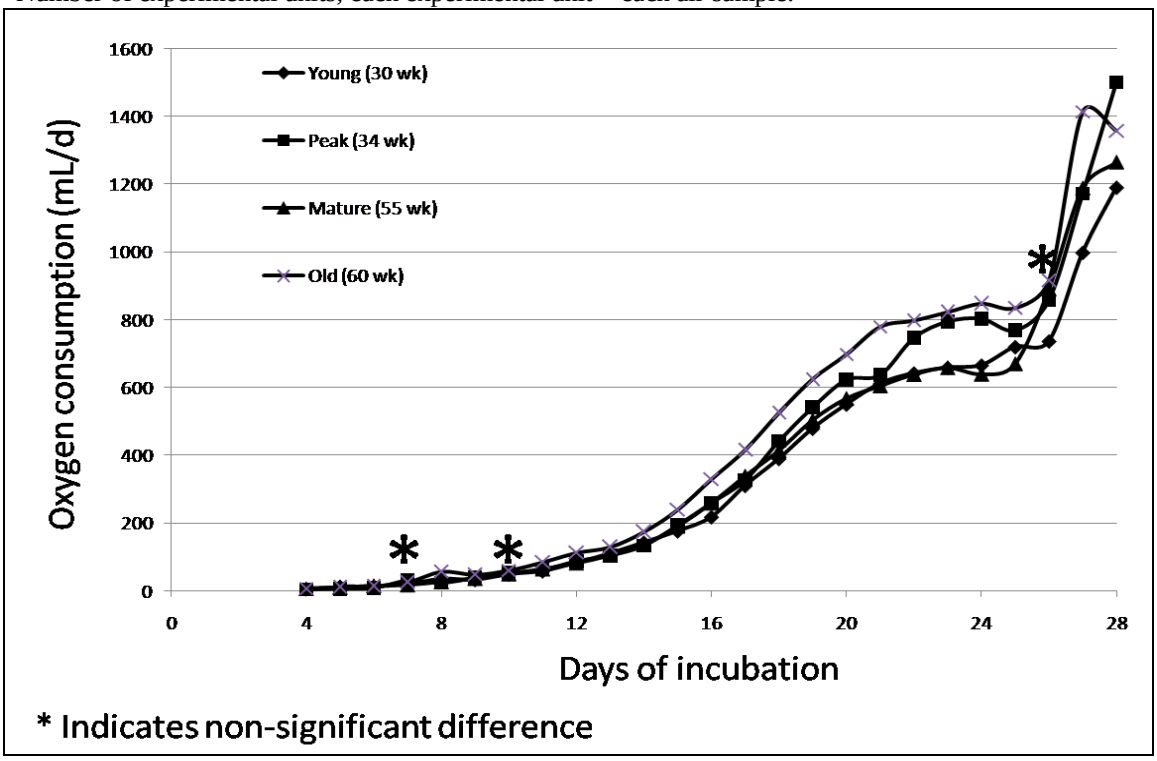

Figure 4.1. Effects of parent flock age on daily embryonic O_2 consumption during 28 days incubation of turkey eggs. Apart from 7, 10 and 26 days of incubation where embryonic O_2 consumption did not differ between flock ages, embryos from old parent flock ages consumed more O_2 from 4 to 28 days.

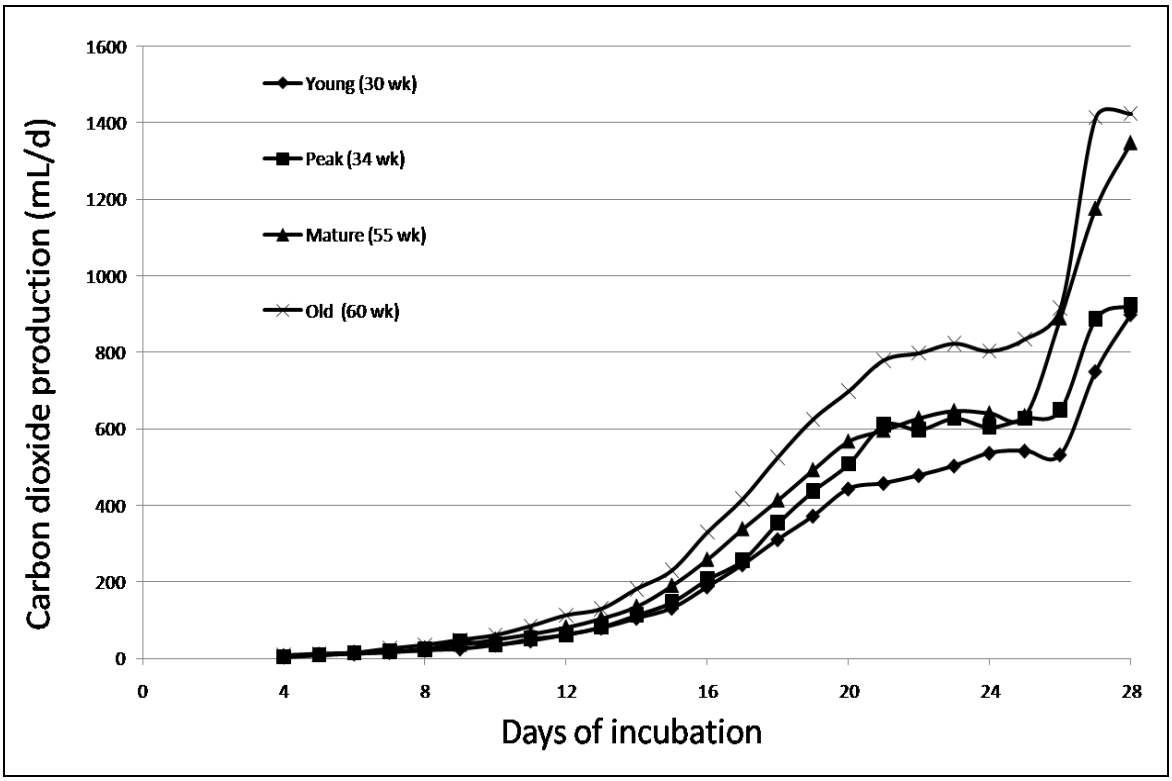

Figure 4.2. Effects of parent flock age on daily embryonic CO_2 production during 28 days incubation of turkey eggs. During all 28 days of incubation starting from 4 days, embryonic CO_2 production was higher in embryos from old parent flock ages than young parent flock ages.

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CHAPTER 5: FLOW CYTOMETRY EXAMINATION OF APOPTOSIS AND NECROSIS: NOVEL TECHNIQUE OF SEPARATING BLASTODERMS[3]

1. INTRODUCTION

Flow cytometry techniques are very popular for the analysis of cell suspensions containing apoptotic and necrotic cells because of their higher sensitivity, greater accuracy, and decreased subjectivity in comparison with other methods such as DNA fragmentation (Liegler et al., 1995). Flow cytometry allows discrimination of cellular populations based on parameters such as cell size (forward scatter), cell complexity (side scatter), and fluorescence which is generally derived from antibody-based labeling of cell surface, cytoplasm or nuclear structures (Foglieni et al., 2001). An accurate assessment of apoptotic and necrotic cells necessitate the maintenance of cell integrity and viability during the isolation and treatment procedures.

The term apoptosis was introduced in 1972 as an active and autonomous type of cell death that does not elicit inflammation (Kerr et al., 1972). Propidium iodide (PI), in combination with Annexin V, provides one approach to distinguish between live, apoptotic and necrotic cells based on differences in membrane permeability (Vermes et al., 1995, 2000). Cells with intact plasma membranes (viable and early apoptotic cells) exclude PI. In contrast, late apoptotic and necrotic cells (non-viable)

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readily stain with PI (Darzynkiewicz et al., 1992; Vermes et al., 1995, 2000). In late apoptotic cells, increased permeability of nuclear pores allows even large proteins and complexes to enter the nucleus (Faleiro and Lazebnik, 2000). In necrotic cells, mitochondrial dysfunction results in the disruption of nuclear membrane integrity (Kroemer et al., 1998). Therefore, in late apoptotic and necrotic cells, PI enters the cell, passes through the disrupted nuclear membrane and intercalates between the double stranded DNA molecules, causing the DNA to glow with red fluorescence in the nucleus (Darzynkiewicz et al., 1992; Vermes et al., 1995, 2000). The capacity to differentiate between apoptotic and necrotic mediated cell death has important implications with regard to surrounding tissue integrity. In short, necrosis results in an uncontrolled release of cellular contents, induction of inflammatory events and associated tissue damage (Zong and Thompson, 2006). In contrast, apoptosis allows for removal of spent cells (e.g. during normal tissue turnover) in the absence of inflammation and tissue damage (Henson et al., 2001).

To investigate apoptosis and necrosis in animal tissues there is a need to develop cell preparation procedures that will maintain cellular integrity. Earlier Petitte et al. (1990) developed a technique to separate chicken blastoderms into individual cells. The blastoderm is the small ‘fluffy’ whitish substance, about 4 mm in diameter found on the surface of the yolk in fertile eggs that develops into the embryo and subsequently the chick. Even though trypan blue exclusion test in this study suggested viability values of greater than 90%, in current study the blastoderm preparations using the method adapted from these authors were associated with significant debris indicating significant cell death that cannot be discerned by the

trypan blue exclusion method. Therefore, it was important to establish a technique that would significantly improve the capacity to maintain cellular integrity of dissociated blastoderm samples. To initially determine the suitability of the technique in achieving the one of the purposes of this thesis, the technique was used to investigate the biological effect of egg storage on cell death mechanisms of apoptosis and necrosis in layer eggs. In the following chapter a further application of the technique will be demonstrated which was the main objective of developing the technique in this thesis, to assess the extent of cell death following egg storage in broiler breeders. This is because cold storage of fertile hatching eggs prior to incubation is a common practice in the poultry industry. However, despite the benefits associated with egg storage, there are significant drawbacks that continue to compromise embryo survivability and net hatchability (Fasenko et al., 2001a, b). Foulkes (1990) as well as Bakst and Akuffo (1999) also found that as egg storage duration increased in chicken and turkey eggs blastodermal cell numbers decreased. However, the exact mechanisms by which egg storage induces loss of blastodermal cells appear unknown. Though Bloom et al. (1998) showed some evidence of apoptosis in chicken blastoderms, the procedure was very subjective as it looked at the blastoderm as a whole. In addition, the use of a single stain to investigate apoptosis appeared to underestimate the extent of cell death reported.

2. RESULTS

2.1. Harvesting and cleaning of blastoderm

Some of the techniques tried in this study and others adapted from previous studies made use of a human hair made into a loop and fitted on a modified glass pipette for blastoderm harvesting and processing (Etches et al., 1996). Unfortunately, the time required completing this harvesting procedure coupled with the constant monitoring under a microscope and user-to-user variations led to significant variability among isolated samples and had a high propensity for blastoderm damage and low cellular yield. In addition, cleaning of yolk from the area opaca proved to be particularly difficult when using this approach. Thus, for the current protocol it was necessary to opt for the use of a 1x $PBS^{-/-}$ drop-wise wash. It was realized that this procedure cleaned the blastoderm and revealed both area pellucida and area opaca (Chapter II, Figure 2.3a) compared to previously shown (Fasenko et al., 2009; Chapter II, Figure 2.3b). Thus, this technique made it easy to examine the whole blastoderm rather than just the area pellucida, as earlier suggested (Petitte et al., 1990). Importantly, this procedure facilitated rapid displacement of the blastoderm off the surface of the perivitelline layer and prevented association of contaminating perivitelline layer and yolk particles in the desired cell suspension, as with previous procedures. The use of hair-loop to remove adhering yolk particles that come in direct contact with the blastoderm was avoided in this study. Also no special instrument was required to separate the blastoderm from the yolk membrane except dropping the $PBS^{-/-}$ on the blastoderm and around it from glass pipette.

2.2. Cell dissociation procedure

Comparison of current dissociation technique and previous ones (Petitte et al., 1990) indicated that the current technique resulted in significantly lower amounts of cellular debri (Chapter II, Figure 2.3c versus 2.3d). It was found that inclusion of an additional discontinuous Percoll gradient step further decreased debri appearance (Figure 5.1), but it was a choice not to include this step during flow cytometry analysis to use debri as an indicator of protocol impact on cellular integrity. Treating the cells with Percoll also increased the yield of viable number of cells, however, this did not reflect on the true nature of the cell suspension since Percoll also removed most damaged cells and thus increased the percentage of cell viability and may have removed some apoptotic and necrotic which were to be examined in the study. While the concentration of trypsin/EDTA solution used was the same as in other studies, the incubation time was increased to 20 min compared to 10 min used by Petitte et al. (1990). However, this did not increase debri compared to other methods because some more frequent practices such as pipetting and vortexing procedures, which likely added to cellular damage in the previous technique were not included. This study showed that blastodermal cells could measure between 25 µm and 125µm in diameter (Figure 2.3d). Foulkes (1990) previously reported cell sizes that ranged from 10 to 50 µm, suggesting that reduction of mechanical disruption through my procedure may have preserved the integrity of larger cells.

2.3. Comparison of live, apoptotic and necrotic cells in layer eggs

Figure 5.2 shows the method of data acquisition and separation of blastodermal cells into live (annex V-/PI-), early apoptotic (annex V+/PI-), necrotic (annex V-/PI+) and late apoptotic/necrotic cells (annex V+/PI+) using flow cytometry. These results indicate that between short-term and long-term egg storage treatments viable embryonic cell numbers decrease with increasing egg storage duration (4 d = 83.58 ± 2.15%; 14 d = 71.42 ± 3.36%) (Figure 5.2). However, the percentage of viable cells in unstored eggs was lower than expected (24.29 ± 8.24%). After repeating cell dissociation and analysis of blastoderms from unstored eggs five times it was realized the results were the same. Since these are primary cells, it is possible that the cells were still dividing when they were used in the study. Konishi and Kosin (1974) revealed that because blastoderms that were harvested soon after oviposition were at the hen's body temperature, they were still undergoing active stages of mitosis. In this study the authors reported that all major mitotic stages were still present in blastodermal cells from unstored eggs but in eggs stored for 2 or 4 d these activities stopped at metaphase. The presence of multiple nuclei in cells from unstored eggs (Figure 5.3) was also attributed to active cell division by Foulkes (1990). It can be suggested that cell cycle events in unstored eggs may have increased cell membrane permeability to PI or make the cells fragile and increased their propensity for mechanical damage associated with cell dissociation procedure. However, it is not known exactly why more cells from unstored or freshly laid eggs had higher percentage of necrotic cells and this may require further investigation.

Importantly, Asmundson (1947) demonstrated that the hatching ability of eggs which were allowed to cool before incubation was higher than that for eggs incubated immediately after laying. Concurrently, increasing egg storage duration beyond the optimum period allowed for storage at cold temperatures (4 d to 7 d) induces significant negative effects in the blastoderm and the entire egg (Bakst and Akuffo, 1999; Fasenko et al., 2009). These effects include but are not limited to some apoptosis (Bloom et al., 1998) and necrosis (speculated by Bakst and Akuffo, 1999).

In the current study, the percentage of early apoptotic cells did not differ significantly between 4 and 14 d stored treatments but it was significantly higher in both stored eggs compared to unstored eggs (Figure 5.2). Since mitosis is reported to stop by 4 d during storage (Konishi and Kosin, 1974) the unfavorable growth arrest by 4 d may contribute to apoptosis induction. The percentage of necrotic cells (Figure 5.2) was significantly higher in unstored eggs (73.56 ± 8.93%) compared to eggs stored for 4 d (3.56 ± 1.64%) and 14 d (16.75 ± 1.73%) (Figure 5.2). In addition, the eggs stored for 14 d had higher necrotic cells than eggs stored for 4 d. Importantly, the fluorescent labels utilized in this study made it easy to move away from morphological features such as nuclear fragmentation, which are not amenable for polymorphonuclear cells (Figure 5.3). Foulkes (1990) reported that under the fluorescent nuclear marker 33258 Hoechst, some larger cells in the blastoderms of incubated unstored eggs appeared to be polynuclear, consisting of either a single multi-lobed nucleus or of several centrally positioned closely situated nuclei. Emanuelson (1965) earlier explained that these multinuclear cells aggregate into

small populations of not more than 30 cells per blastoderm and are most frequently located at the periphery of the intact blastoderm. These cells were suggested to be the result of divisional abnormalities, probably occurring as result of O_2 shortage in the blastoderm as the egg traverses the inter-uterine canal (Emanuelson, 1965). The percentage of late apoptotic/necrotic cells was significantly higher in 14 d stored eggs (7.36 ± 1.53%) than 4 d (2.31 ± 1.52%) and unstored eggs (1.12 ± 1.39%) (Figure 5.2). Since cells in late apoptosis and or necrosis are already dead, they contribute to a major non-viability of the embryo. Besides necrosis cell death can result in proinflammatory responses which can be deleterious to neighboring cells.

2.4. Proving evidence of apoptosis and necrosis

An examination of the morphology of isolated blastodermal cells using the ImageStream multispectral flow cytometer was used to provide evidence on the occurrence of apoptotic and necrotic cell death. Since the ImageStream multispectral flow cytometer can allow for increased spatial resolution and examination of morphological changes, cells from all egg storage treatments were also examined for the purposes of comparing data. It was observed that the results were similar to the measurements from classical flow cytometer (data not shown). Figure 2.6 shows the process of separating blastodermal cells into viable, early apoptotic, necrotic and late apoptotic/necrotic cells under the ImageStream multispectral flow cytometer. The results also provide evidence of the occurrence of apoptosis and necrosis according to differential absorption of either annexin V or PI dye (Figure 2.6) and are a

representative demonstration of what types of cells are present in all egg storage treatments.

3. DISCUSSION

Because chicken blastoderms are very small (only about 4 mm in diameter), fragile and they be difficult to separate. This difficulty probably encouraged earlier researchers to apply pipette dispersion (Aritomi and Fujihara, 2000), enzymatic and chemical disruption techniques (Petitte et al., 1990; Etches et al., 1996) during the separation of blastodermal cells. From the current study, it appears that previous researchers overemphasized the sticky nature of chicken blastodermal cells and subjected the blastoderms to over-pipetting and vortexing resulting in cellular breakage. It is important that one is able to thoroughly clean the blastoderm prior to dissociation to reduce debri resulting from yolk particles. However, the drop-wise release of fluid over the blastoderm was performed carefully so not to disperse the blastoderm. Based on previous experiences, 40 blastoderms were pooled together in order to include a number of steps aimed at reducing debri and clumps resulting from doublets generated in the cell suspension. While flow cytometry acquisition did not necessarily require such large numbers of blastoderm, it was important in my technique where a significant fraction of cells were lost through grading between single, giant and doublet cells which when maintained could have affected results.

During embryonic development in fruit fly, *Drosophila melanogaster*, multiple forms of cell deaths have been identified. These included apoptosis (type I programmed cell death (PCD)), autophagy (type II PCD), nonlysosomal cell

disintegration (type III), necrosis, necroptosis (characterized by necrotic cell death morphology and activation of autophagy) and endoplasmic reticulum (ER) stress-induced cell death (Díaz et al., 2005). However, it was important to focus on establishing the biological importance of apoptosis and necrosis in this study because these are very important mechanisms that have been previously postulated to affect embryonic development during egg storage. But they have not been investigated in detail, either has there been a clear cut argument to show that these cell death mechanisms actually occur during blastoderm development prior to incubation.

The study showed that storage of layer hatching eggs reduces the viability of blastodermal cells based on annexin V and PI assays. Even though the blastodermal cells at this stage are undifferentiated a significantly higher amounts of necrotic and late apoptotic/necrotic cells in 14 d compared to 4 d stored eggs were observed. These numbers could have a potentially harmful effect on development of a blastoderm from eggs stored for 14 d when a favorable environment (temperature) is provided after egg storage (Fadok et al., 2001). Necrosis by definition can result in significant tissue damage. The higher levels of necrotic cells in eggs stored for 14 d can have a long term effect on other viable cells during incubation as result of release of inflammatory responses. This could be the cause of lowered productivity in eggs stored for 14 d compared to 4 d as reported in earlier studies. With reference to the results of the current and earlier studies (Bakst and Akuffo, 1999), cold storage induction of apoptosis and necrosis as well as the concomitant reduction in viable blastodermal cell numbers could be linked to previously observed decreased

chicken embryo survival (Elibol et al., 2002), slowed embryo development (Mather and Laughlin, 1977; Fasenko et al., 2001b), decreased hatchability (Fasenko et al., 2001b) and increased number of embryonic abnormalities and mortality (Mather and Laughlin, 1977; Fasenko et al., 2001b) in eggs cold stored for 14 d vs. eggs stored for 4 d. However, most of these studies were conducted with broiler breeder eggs and therefore may require an independent study in broiler breeder egg to fully support the suggested relationship.

Through flow cytometry analysis (FACScan) it was discovered that significant amount of debri were still captured until an ImageStream multispectral flow cytometer was used to validate the data. Although, there are other complex and conventional types of flow cytometer that can be used, the availability of the ImageStream multispectral flow cytometer made acquisition of results easier. However, initial attempts to sort the cells and represent the morphological characteristics of viable, apoptotic and necrotic cells under low power microscope were not successful because of low population of cells from necrotic and late apoptotic/necrotic cells. Also DNA fragmentation method was not successful possibly due to the small amount of DNA that results from isolated blastoderms and insensitivity of this technique. All these practices later necessitated the use of more blastoderms in order to obtain large cell population for subsequent studies. However, later use of ImageStream multispectral flow cytometer though provided more success may not be very reliable in all experimental cases because there are just a few around the world. It is also expensive to analyze one sample, complex to operate, and requires an expert's operation. But at present, it appears to be the

golden key to proper cell separation and quantification into live, apoptotic and necrotic cells because it is more advanced than classical flow cytometry analysis.

4. Summary and conclusion

Since factors such as genetic selection, age of breeder strain, fertile egg storage and incubation temperature can affect embryonic development the above technique can become useful to study the effects of these factors on survival of individual blastodermal cells. In addition, the technique described will be necessary to examine cell cycle events in individual blastodermal cells. For example in one of the labs where some of the experiments in this thesis were conducted is currently trying to investigate the minimum temperature required for cell cycle events to occur in embryonic cells (physiological zero). This can afford poultry physiologists aiming to investigate the physiological zero of individual cells the tool to finally settle the debate on which temperature is the most favorable to store eggs for optimum performance, thus the technique can be used to obtain single cells for such study. In addition, scientists seeking to improve the development and commercialization of chimera birds (Teillet, 2008) have a better opportunity to increase the percentage of viable cells collected from the donor blastoderm. Some studies have revealed that there is a correlation between apoptosis and cell phenotype (Carbonari et al., 1995). The investigation of this relationship ideally requires techniques that permit the concomitant detection of apoptosis and cell phenotype analysis at a single cell level. The current study has thus opened a wide door for similar studies that seeks to characterize genes that catalyses apoptosis and or necrosis during eggs storage.

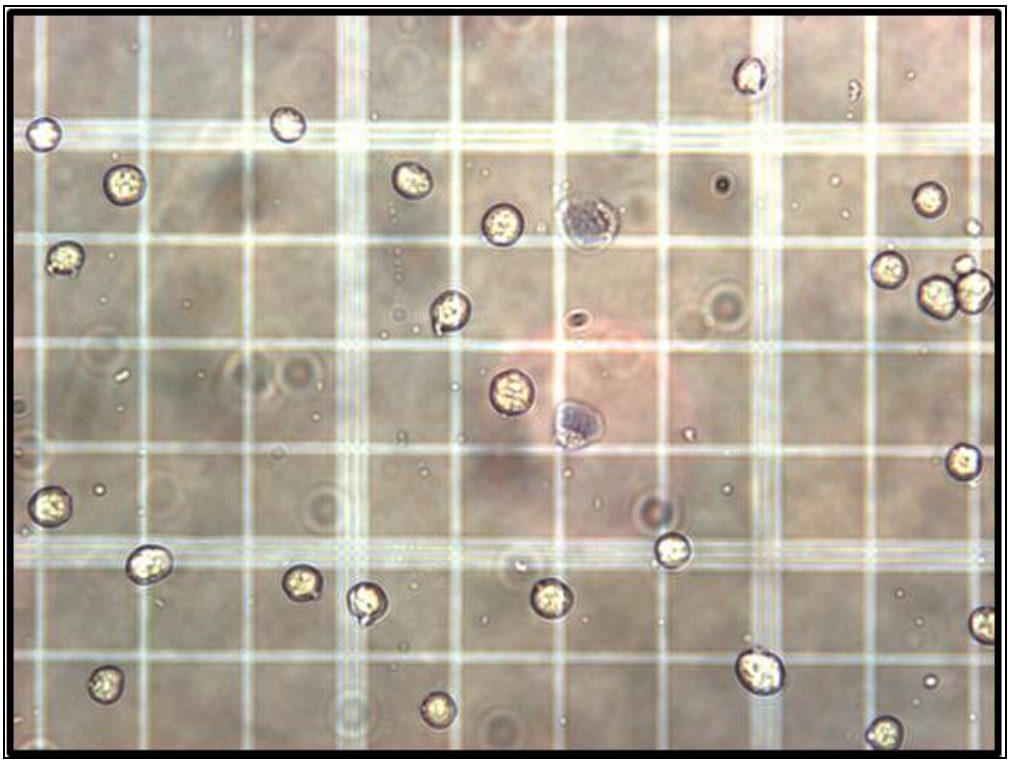

Figure 5.1. Representative sample of blastodermal cells treated with Percoll to demonstrate a decrease in debri appearance (20x magnification)**.**

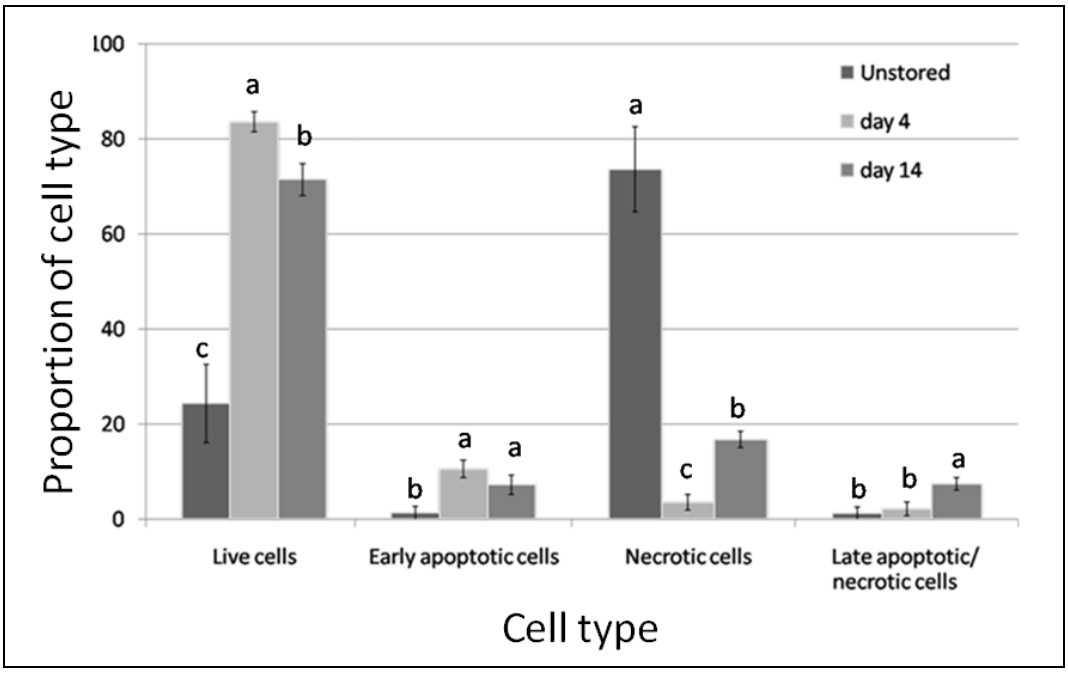

Figure 5.2. Effect of egg storage duration on the percentage of viable and non-viable blastodermal cells in layer eggs stored for 0, 4 and 14 d. The cell population prepared from each storage treatment was distributed by flow cytometry (BD FACScan) into a) live (AnnexV- / PI-), b) early apoptotic (AnnexV+ / PI-), c) necrotic cells (AnnexV- / PI+), and d) late apoptotic/necrotic cells (AnnexV+ / PI+) (n = 4), where n = number of independent experiments which were repeated. For each experiment 35 to 40 blastoderms were pooled together for analysis on the flow cytometer. Significant differences were separated by the PDIFF procedure of SAS ($P \leq 0.05$). The proportion of blastodermal cells from all storage treatments were compared for a) live (AnnexV- / PI-), b) early apoptotic (AnnexV+ / PI-), c) necrotic cells (AnnexV- / PI+), and d) late apoptotic/necrotic cells (AnnexV+ / PI+). The data show that the viability of unstored egg cells is not good which is consistent with the poor performance of unstored eggs when incubated (Asmundson, 1947). In addition, increasing egg storage duration from 4 d to 14 d decreased the viability of blastodermal cells and increased both necrosis and late apoptosis/necrosis events.

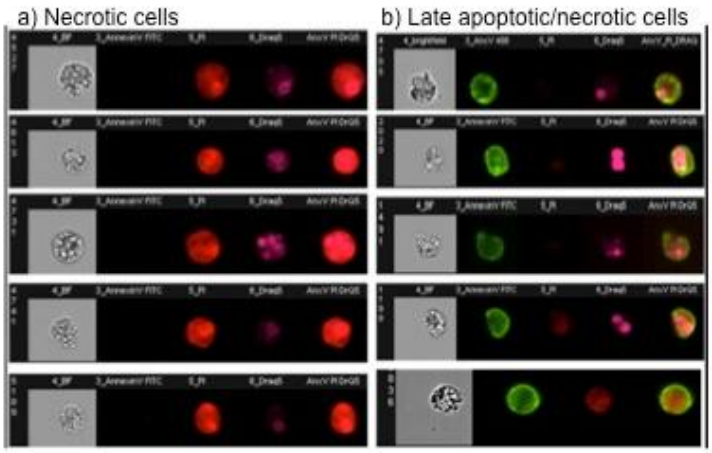

Figure 5.3. Representative sample of blastodermal cells isolated from unstored chicken eggs. Necrotic cells contain multiple nuclei, an evidence of cell division or duplication as suggested by Konishi and Kosin (1974). The presence of two nuclei within cells group classified as late apoptotic/necrotic cells could be due to the formation of apoptotic bodies which marks the completion of apoptosis cell death event or mitotic cell division, a characteristic feature of most of blastodermal cells isolated from unstored eggs.

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CHAPTER 6: EFFECTS OF BROILER EGG STORAGE ON EMBRYO QUALITY: EMBRYONIC METABOLISM, CELL VIABILITY AND EXPRESSION OF APOPTOSIS GENES[4]

1. INTRODUCTION

Cold storage of fertile hatching eggs is a common practice in the poultry industry. In Canada, broiler-hatching eggs are usually stored for short periods (3 to 7 d) in the farms and/or hatcheries before incubation since most breeder farms are located at significant distances from the hatchery. Other logistic problems such as limited incubator space and the sake of maximizing incubator energy sometimes compel farmers and hatcheries to store the eggs for periods beyond 7 d. However, in the poultry industry, storage duration longer than 7 d has had significant negative effects such as decreased hatchability (Fasenko et al., 2001b) and increased embryonic mortality (Walsh et al., 1995; Fasenko et al., 2001b), although from management point of view the longer period practice helps to effectively manage the large size of eggs collected. In addition, embryos from broiler eggs cold stored until 14 d display reduced growth rate and poor chick quality compared to eggs stored for 4 d (Fasenko et al., 2001b). In other studies, it has been identified that the storage process can affect qualities of yolk and albumen, perivitelline membrane, and blastoderm, gas exchange and embryonic metabolism, and egg moisture loss (Bakst

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and Akuffo 1999; Lapão et al., 1999; Fasenko, 1996; 2007; Fasenko et al., 2002, 2009). Recent studies have also reported that egg storage practice can cause blastodermal cell death like activation of apoptotic (programmed cell death) and necrotic (injurious cell death) cell death mechanisms (Hamidu et al., 2010). Whereas some researchers suggest that certain manipulations of the eggs before and during storage may be appropriate to reduce embryonic mortality (Fasenko et al., 2001b), increase embryo viability or egg quality as a whole (Fasenko, 2007), many hatcheries have not adopted the practices because the fundamental reasons for these suggestions have not been proven. Though this study and past ones advised farmers and hatchery managers not to store eggs beyond 7 d based on some of the consequences listed above and other reasons unknown, in broiler breeders, a fundamental understanding of the effects of egg storage on embryos at the metabolic, cellular and molecular levels have received limited attention (Foulkes, 1990; Bloom et al., 1998; Bakst and Akuffo 1999; Hamidu et al., 2010).

In chickens, a healthy embryo (blastoderm) contains averagely 60,000 embryonic (blastodermal) cells following oviposition (Petitte et al., 1990; Etches et al., 1996). However, it has been hypothesized that a minimum number of healthy blastodermal cells may be required to initiate normal embryonic development (Fasenko et al., 1992). The authors further suggested that long-term cold storage practice might have decreased healthy embryonic cell numbers below a critical threshold that prevents normal embryo development, induces abnormal development or increases embryo death in 14 d stored eggs (Fasenko et al., 1992). Though in a

recent study, we showed that an increased activation of apoptosis and necrosis cell death in layer blastoderms was associated with a reduced percentage of viable blastodermal cell numbers following egg storage this fact has not been established in broiler breeder eggs (Hamidu et al., 2010). Broiler breeders are quite different from layers by the intensity of genetic selection they have undergone and may not have the same amount of cell deaths identified in layer eggs. In addition, the exact molecular mechanisms that may have led to the cell death layers (the genes expressing apoptosis) may not be the same in broiler breeder embryos. Therefore, we hypothesized that an increased activation of blastodermal cell death following egg storage may be one of the cellular and/or molecular mechanisms leading to reduced embryonic metabolism and embryonic development in broiler breeder embryos reported earlier (Fasenko et al., 1992). To understand the effects of storage duration on embryonic development, we investigated embryonic metabolism, blastodermal cell viability and expression of selected apoptosis genes under two storage conditions (4 d versus 14 d). The current study may have the potential to supply the information needed to prevent some of the adverse effects of prolonged egg storage and/or understand the fundamental reasons why broiler eggs should not be stored for a longer period.

2. RESULTS AND DISCUSSION

2.1. Embryonic and chick performance

Initial egg weight before storage was higher in 4 d versus 14 d stored eggs. However, after covariance analysis of initial egg weight before storage on final egg

after storage chick weight and chick carcass analysis, the final egg weight after storage and chick weight were still higher in 4 d versus 14 d stored eggs (Table 6.1). The chick weight was expected because embryos from 4 d stored eggs developed better than 14 d stored eggs. The time taken to pip and to hatch was not different between storage duration after covariance analysis. This result disagrees with earlier results (Christensen et al., 2001; Fasenko and Robinson, 1998; Tona et al., 2003). The effect of storage was very noticeable on egg weight as well as evident in the development of the embryos (wet and dry embryo weight) and chicks (live weight) that hatched. However, there were no differences in the egg components measured and chick carcass characteristics such yolk free body weight and residual yolk sac weight (data not shown) confirming that the effect of storage is more critical on the developing embryo and the chick which is product hatching egg producers are expected to deliver compared to carcass components which are mostly important for research purposes..

Storage had significant effect on daily embryonic body weight (Table 6.2). Wet weight of embryos from 4 d stored eggs was significantly higher than that of 14 d stored eggs throughout the measurement period except 18 days of incubation. Dry body weight of embryos was also significantly higher throughout incubation in 4 d stored eggs versus 14 d stored eggs, except for 4, 14, 18 and 20 d of incubation where no differences were observed. These results were consistent with data reported by Christensen et al. (2002), where prolonged storage reduced daily embryo body weight. Other studies have reported similar lower embryo body weight when egg storage duration exceeded 8 d (Yalcin and Siegel, 2003). Reduction in embryo

body weight may be due to a slowdown in metabolic activities, which utilize yolk nutrients for growth of the embryos.

2.2. Embryonic heat production

As expected, there was a significantly higher daily EHP in 4 d versus 14 d storage groups (Table 6.3). Particularly, embryos from 4 d stored eggs produced higher amounts of heat on d 14, 16, 17 and 20 of incubation compared to 14 d stored eggs. The higher heat production was associated with higher consumption of O_2 and higher production of CO_2 and may have resulted from larger percentage of active embryonic cells. Therefore, the reduction in embryonic body weight previously observed (Table 6.2) in 14 d stored eggs could also be attributed to limited metabolism of these embryos as a result of fewer than normal cell numbers, which may have resulted from death of some embryonic cells (Hamidu et al., 2010) and thereby reducing the total viable or metabolizing cells (Fasenko, 2007). This suggestion agrees with previous studies also suggesting that a disturbance in oxidative metabolism can reduce embryo proliferative capacity under suboptimal environmental conditions, which under the current study is egg storage (Emanuelson, 1965; Leese, 1998; Thompson et al., 2002; Fasenko, 2007). Based on the results reported above, it was easy to moved confidently to characterize the mechanistic basis for decreased performance (i.e. embryo weight and metabolism) in 14 d stored eggs.

2.3. Cell viability and cell death

Results from this study showed that percentage of live cells was significantly higher in eggs stored for 4 d (81.17 ± 2.15) compared to eggs stored for 14 d (68.18 ± 2.13) ($P < 0.0001$) (Figure 6.1 A). The result is consistent with our previous study where viable embryonic cell numbers decreased when egg storage duration increased from 4 d to 14 d (Hamidu et al., 2010). Other studies have found similar trends (Bakst and Akuffo, 1999; Foulkes, 1990; Bloom et al., 1998). Therefore, we suggest that these changes in embryonic cell numbers may be responsible for the poor embryonic performance during incubation observed in other studies (Mather and Laughlin, 1977; Fasenko et al., 1992, 2001b; Fasenko, 2007).

In a previous study it was observed that a reduction in blastodermal cell numbers was primarily due to increased amounts of cell death from apoptosis and necrosis (Hamidu et al., 2010). While in the previous study the kind of cell deaths identified were the extremes of apoptosis (late apoptosis) and necrosis (often associated with release of inflammatory responses such as toxins), in the current study only the early stage of apoptosis was observed to make a difference. The percentage of early apoptotic cells was significantly higher in 14 d (17.88 ± 1.87%) versus 4 d (4.32 ± 1.89%) storage treatments ($P < 0.0001$). The percentages of necrotic cells and late apoptotic/necrotic cells were not significantly different between eggs stored for 4 d (10.86 ± 0.76%; 3.38 ± 0.56%) and 14 d (10.48 ± 0.75%; 3.38 ± 0.55%) respectively (Figure 6.1 A). During early stages of apoptosis, one of the plasma membrane alterations at cell surface is the translocation of the phosphatidylserine, a phospholipid-like cell membrane protein that is normally

located on the inner side of the cell membrane to the outer membrane, a physiological signal indicating that apoptosis has started. During the initial stages of apoptosis the cell membrane remains intact, which is different from other forms of cell death such as late apoptosis and necrosis where there is loose of cell membrane integrity and the cell may become leaky (Vermes et al., 1995). Though apoptosis has a healthy physiological function for the embryo, an important mechanism responsible for removing unwanted and defective cells in order to maintain tissue hemeostasis (Kerr et al., 1972), the approximately 18% of early apoptosis observed in broiler eggs stored for 14 d in the current study may have additional effect on embryonic development which needs to be taken seriously. This is because once cells have begun the process of apoptosis, they will eventually proceed to late apoptosis where the cells divide into apoptotic bodies and may be taken up by neighbouring cells. In blastoderm this mechanism may serve to reduce the number of viable embryonic cells that may be important for critical embryonic development during incubation. Though the current study examined cell death at a much wider scope than previous studies (Foulkes, 1990 and Bloom et al., 1998), it also appears to be the first study to actually provide sufficient data to support the hypothesis that broiler egg storage reduces viable blastodermal cell numbers leading to reduced embryonic development measured by embryo weight and metabolism (Fasenko et al., 1992).

Physiologically, a reduction in blastodermal cell numbers may also reduce the amount of O_2 that each embryo can take because of reduced metabolism observed above. Even if O_2 is available, a reduction in embryonic metabolism suggests that

the embryos do not have enough cells to make effective use of the available O_2 to breakdown molecules of carbohydrates, fats or proteins to release the needed energy for embryonic growth (Table 6.2). Between apoptosis and necrosis, recent study suggests that the potentials of controlling the event could be higher than necrosis because its exact sequence of molecularly regulated events is well known than necrosis (Fabbri1 et al., 2006). Normally, when apoptosis reaches the late stages where cells have already divided into apoptotic bodies, it is acceptable that the process is irreversible. However, some studies suggest that cells can recover, but only if they have not reached the late stage of apoptosis, where they can be engulfed by nearby phagocytotic cells (Green and Beere, 2001). It has been reported that for cells in which caspases (enzymes that executes apoptosis) have been activated, they still progress through a state of being 'mostly dead', a stage that physically resembles the early phase of apoptosis but from which cells can fully recover (Reddien et al., 2001; Hoeppner et al., 2001). Since apoptosis, unlike necrosis, has well known molecular basis, an understanding of the molecular mechanism may provide information on the expression levels of which apoptosis genes can influence cell death during egg storage so that the knowledge can be used to advise broiler hatching egg producers and hatchery manager why they should not over store the eggs or it can help embryologists design future experiments to reduce some of the negative impacts of prolonged egg storage (McKenna et al., 1998).

Previously, necrotic cell death was traditionally considered a passive and chaotic process; however, emerging evidence indicates that specific molecular mechanisms may trigger cellular destruction during necrosis (Tavernarakis, 2007;

Hitomi et al., 2008). Therefore, it is not conclusive that there is no molecular basis for necrotic cell death. However, apoptotic cell death has been widely investigated particular genes also direct this kind of cell death have been characterized. In addition, the assays for studying apoptosis are available to begin a preliminary study like the current investigation. Since this was preliminary study only a few apoptotic genes were selected to determine to what extent broiler egg storage influences gene expression.

2.4. Suitable housekeeping genes and PCR efficiency of target genes

The table 6.4 shows the procedures that were used to determine that the housekeeping genes were suitable as normalization genes for the target genes. Through statistical analysis it is evident that there were no significant differences between the Ct values of the 4 d and 14 treatments at the level of the broiler embryos incubated for 6 d. Therefore it was concluded that the Ct values could be averaged as normalization factor. However, for broiler blastoderms, the Ct values were significant; therefore the coefficient of variation (CV) was used. Previous study shows that a CV of less than 10% could be reliable to choose a gene for housekeeping purposes (Chen et al., 2010). Since all three genes under consideration had CV values far less than the 10% they were considered suitable in the current study. In addition, the average Ct values were about only one to two cycles or a fraction from one treatment to the other. The results from the PCR efficiency values show that the primes used for the study amplified the DNA samples correctly (Table 6.5). The PCR efficiency has a major impact on the fluorescence history and the

accuracy of the calculated expression result and is critically influenced by PCR reaction components (Pfaffl, 2004). The author further explained that individual samples generate different and individual fluorescence histories in kinetic RT-PCR or during the PCR process. And that the shapes of amplification curves differ in the steepness of any fluorescence increase and in the absolute fluorescence levels at plateau phase depending on background fluorescence levels. In addition, degradation in the DNA used will result in bad PCR efficiency values. Another criterion used to verify the accuracy of the PCR process was a gel electrophoresis of the qRT-PCR products. In this study it has been shown that all the primers amplified the genes to the correct product sizes corresponding to 50 to 70 bp which were expected from the RT-PCR reactions (Figure 6.2). The study shows that the DNA samples and primers used were correctly isolated or designed respectively to amplify the selected genes as shown in chapter 2 (Table 2.1).

2.5. Gene expression in broiler blastoderms

The expression of pro-apoptotic genes (*Bak*, *Bax* and *Bok*) increased by 20, 3.4 and 7 fold respectively in broiler blastoderms isolated from eggs stored for 4 d comparing to those from eggs stored for 14 d (Table 6.4; Figure 6.1 B). The expression of anti-apoptotic gene *Bcl-2* was similar between storage treatments (1.19 fold) but *Bcl-xL* gene, a homolog of *Bcl-2* was slightly down-regulated (0.85 fold) as egg storage duration increased from 4 d to 14 d. The occurrence of apoptosis in broiler blastoderms appears to be dependent on the increased expression of the pro-apoptotic genes *Bak*, *Bax* and *Bok*. The expressions of either the pro-apoptotic or

anti-apoptotic genes are very important events during apoptosis. It is normally expected that for apoptosis to proceed, environmental effects such as temperature can cause the expression of the pro-apoptotic genes to increase above that of the anti-apoptotic genes (Yin et al., 1997; Ikeda et al., 1999; Myers et al., 2007; Osborne et al., 2008). However, when the antiapoptotic genes levels increase they lead to cell survival by protecting the cell. Other studies have confirmed that higher expression of *Bax* in particular over *Bcl-2* resulted in the cells sending a death command signal but when *Bcl-2* dominated, cell death was inhibited at the expense of cell survival (Korsmeye, 1999; Suyama et al., 2001; Walsh et al., 2008).

Practically, the anti-apoptotic gene duo, *Bcl-2* and *Bcl-xL* and their associated proteins form a ‘mesh’ of ‘protective layer’ over the organelles in the cell, especially the mitochondria from the action of the pro-apoptotic proteins (Ma et al., 2001). From the results of the current study, we can state that the increased expression of the pro-apoptotic genes (*Bak, Bax* and *Bok*) over the anti-apoptotic genes (*Bcl-2* and *Bcl-xL*) as egg storage duration increased from 4 d to 14 d may have induced this chain of cell death mechanism and resulted in the increased percentage of apoptotic cells and eventual reduction in viable blastodermal cells. Since the mitochondria, the “metabolic house” of the cell is the main target point of cell destruction it is not surprising that for the treatment that had more apoptosis (14 d) it also had more embryos with lower body weights and reduced metabolism than 4 d stored eggs.

2.6. Gene expression in embryos from incubated broiler eggs

The expression level of all genes investigated was down-regulated in embryos at 6 d of incubation (Table 6.4; Figure 6.1 B). While the downward regulation of the anti-apoptotic genes *Bcl-2* and *Bcl-xL* were consistent with the current research objectives, it was expected that the expression of the pro-apoptotic genes would increase rather than decrease. It is not clear if the heat of incubation may have deactivated apoptosis genes or denatured their proteins that resulted in the downward regulation of all genes (Chen et al., 2008). Since the expression of apoptotic proteins was not measured in the current study, future studies should examine protein expression to confirm if observations made at the genetic level are related to protein expression.

Although storing eggs up to 14 d has its own negative implication on the embryo, some of the embryos developed to the time of hatching. In the current study, it was noticed that the embryos from 14 d stored eggs hatched with lower body weight chicks compared to chicks from 4 d eggs (Table 6.1). This could be an indication that the biological effect of apoptosis associated with long-term cold storage is still evident before and after the embryo hatches. We hypothesize that heat treatment of eggs may have prevented apoptosis to some extend due to its deactivation of all apoptosis genes studied. Previous results appear to support this hypothesis. Fasenko et al. reported that when eggs were pre-warmed for 6 h before storage, the embryos developed to the hypoblast stage, (embryonic cells differentiate into endoderm, ectoderm, and sometimes hypoderm) and this resulted in higher hatchability of the pre-warmed eggs stored for 14 d compared to those not pre-

warmed (Fasenko et al., 2001b). Similarly, Foulkes (1990) reported that a reduction in blastodermal cell numbers were lower in egg stored at higher temperatures compared to lower temperatures. The results of the current study and those from previous researchers indicate that future manipulation of storage practices and storage temperatures such as pre-heating eggs before storage may help to increase hatchability of long-term stored eggs.

Based on the data shown, egg storage appears to influence embryonic development right after oviposition and during incubation. Since genes control physiological events such as metabolism, egg storage should not only be investigated at the level of the blastoderm but also at the level of the developing embryo. At this level it is possible to reveal some of the basis for poor embryonic development. Since the expression of some of apoptosis genes have been implication in poor embryonic growth, more attention should be focused on gene regulation to determine if it can be used to improve embryo vitality. Currently, a gene like eukaryotic initiation factor 5A (eIF-5A) has been identified in some higher animals and plants to minimize apoptosis and improve survivability (Caraglia et al., 2003; Hopkins et al., 2008). An intensive study of such a gene in poultry species may have the potential to limit the amount of apoptosis that occurs during cold storage. The investigation can be extended to identify broiler breeder eggs that are resistant to cold storage. Recently I had a discussion with a Lohmann chicken staff, and it appeared that some layer strains may have the ability to resistant prolonged egg storage to some length just by visual examination of the blastoderm but this is yet to

be scientifically proven “C. Robert (Lohmann Tierzucht Gmbh, Am Seedeich, Cuxhaven, personal communication)”.

3. Summary and conclusions

Our study shows that longer storage duration of broiler breeder eggs led to reduced embryonic metabolism, viable embryonic cell numbers, increased early apoptosis events such as increased expression of pro-apoptotic genes and reduced expression of anti-apoptotic genes at the blastodermal cell level. Since apoptosis is a genetically regulated event, it can be controlled. Therefore, additional research is needed to focus on reducing the induction of apoptosis by cold storage up to 14 d. Studying the genes that execute apoptosis and prioritizing effective mechanisms that can control their actions should be the next direction to increased embryonic survival in the future. The current study has provided some potential insights into why egg storage reduces hatchability and increases embryonic mortality. We hope that the findings of this study can also lead to the discovery of new broiler breeder strains that can withstand the impact of apoptosis due to cold storage and increase embryo survival during egg storage and incubation.

Table 6.1. Effect of egg storage duration on egg weight and chick performance

Storage Treatment	Egg weight before storage[1] (g)	Egg weight after storage[2] (g)	Chick weight[3] (g)	Pipping time[4] (h)	Hatching time[5] (h)
4d	61.59[a]	61.01[a]	46.56[a]	494.55	506.81[a]
14 d	61.06[b]	60.56[b]	44.32[b]	473.57	487.28[b]
SEM	1.06	0.07	1.15	23.68	20.39
P-value	0.0141	<0.0001	0.0054	0.4220	0.4231

[a,b]Means in the same column with different superscripts differ significantly ($P \leq 0.05$).

[1]Egg weight at the time of collection, before storage.

[2]Egg weight at the time of setting into incubator.

[3]Chick weight immediately after hatching.

[4]Time taken from setting eggs in incubator to external pipping.

[5]Time taken from setting eggs in incubator to chick hatched out.

Table 6.2. Effects of egg storage duration on wet and dry embryo weight

Day of incubation	Wet embryo weight[1] (g)			Dry embryo weight[2] (g)		
	4 d	14 d	SEM	4 d	14 d	SEM
4	0.04[a]	0.03[b]	0.01	0.004	0.003	0.001
5	0.14[a]	0.12[b]	0.03	0.008[a]	0.007[b]	0.002
6	0.30[a]	0.25[b]	0.05	0.018[a]	0.016[b]	0.003
7	0.59[a]	0.52[b]	0.19	0.037[a]	0.033[b]	0.006
8	1.01[a]	0.88[b]	0.11	0.07[a]	0.06[b]	0.01
9	1.59[a]	1.44[b]	0.20	0.11[a]	0.10[b]	0.02
10	2.31[a]	2.19[b]	0.30	0.17[a]	0.15[b]	0.02
11	3.32[a]	3.05[b]	0.46	0.26[a]	0.23[b]	0.04
12	5.13[a]	4.71[b]	0.78	0.43[a]	0.40[b]	0.08
13	7.62[a]	6.99[b]	1.30	0.73[a]	0.65[b]	0.18
14	11.47[a]	10.52[b]	1.22	1.18	1.16	0.30
15	14.94[a]	13.57[b]	1.07	2.04[a]	1.77[b]	0.27
16	18.59[a]	17.37[b]	1.76	3.06[a]	2.74[b]	0.40
17	22.85[a]	21.40[b]	1.33	4.00[a]	3.74[b]	0.40
18	27.24	26.37	1.34	4.94	4.77	0.38
19	30.10[a]	29.80[b]	1.23	5.72[a]	5.45[b]	0.30
20	34.00[a]	31.81[b]	1.54	6.42	6.24	0.35
21	37.53[a]	35.89[b]	1.47	7.91[a]	7.33[b]	0.31

[a,b]Means in the same column with different superscripts differ significantly $P \leq 0.05$).

[1]Least square means of wet embryo weight after breaking open 30 eggs (15 eggs/ storage) daily.

[2]Least square means of dry embryo weight after drying the samples in oven for 4 d at 65 C.

Table 6.3. Effects of egg storage duration on daily embryonic heat production

Day of incubation	Heat production (mW)[1]	
	4 d	14 d
4	1.15 ± 0.24	1.22 ± 0.26
5	2.97 ± 1.18	2.77 ± 1.19
6	4.42 ± 0.95	4.13 ± 0.96
7	5.06 ± 1.03	5.11 ± 1.06
8	20.95 ± 1.60	21.61 ± 1.60
9	23.78 ± 1.76	24.01 ± 1.76
10	33.77 ± 5.52	34.29 ± 5.52
11	49.26 ± 4.58	45.44 ± 4.58
12	73.36 ± 9.53	68.83 ± 9.56
13	99.39 ± 7.32	94.98 ± 7.32
14	128.90 ± 6.43[a]	119.90 ± 6.43[b]
15	155.89 ± 5.98	153.15 ± 6.12
16	191.61 ± 7.42[a]	183.17 ± 7.42[b]
17	206.93 ± 4.15[a]	198.17 ± 4.14[b]
18	194.89 ± 9.45	195.85 ± 9.45
19	220.94 ± 8.17	225.49 ± 8.17
20	263.92 ± 12.32[a]	223.46 ± 11.72[b]
21	339.90 ± 26.82	327.30 ± 25.28

[a,b]Means within each day and different superscripts differ significantly ($P \leq 0.05$).

[1] Daily average heat production.

[2]Daily average heat production /daily average dry embryo weight.

Table 6.4. Selecting housekeeping genes base in statistics and coefficient of variation

Sample	Gene	Treatment	Av. Ct[1]	P-value[2]	StdDev[3]	CV[4]	Av. CV[5]
Blastoderm	*HPRT*	4 d	25.39	<0.0001	1.4127	0.0558	0.0497
		14 d	27.81		1.2255	0.0436	
	B-actin	4 d	19.52	0.0011	1.3067	0.0668	0.0589
		14 d	21.80		1.1630	0.0509	
	UBQ	4 d	19.02	0.0058	1.0626	0.0573	0.0565
		14 d	20.21		1.2060	0.0557	
6 d old broiler embryo	*HPRT*	4 d	26.23	0.4361	1.2485	0.0476	0.0501
		14 d	25.47		1.3395	0.0526	
	B-actin	4 d	17.34	0.4635	1.1176	0.0645	0.0635
		14 d	17.87		1.1165	0.0625	
	UBQ	4 d	18.59	0.1936	1.1501	0.0619	0.0612
		14 d	19.57		1.1825	0.0604	

[1]Av. Ct = average threshold cycle numbers from triplicate wells.

[2]*P*-value = statistical significance.

[3]StdDev = standared deviation.

[4]CV = coefficient of variation.

[5]Av. CV = average coefficient of variation.

Table 6.5. Real time PCR amplification efficiency

Gene	Days of storage	Broiler blastoderm	6 d Old broiler embryo
Bak	4 d	1.14	1.18
	14 d	1.01	1.11
Bax	4 d	0.86	1.00
	14 d	1.21	0.93
Bok	4 d	0.98	1.07
	14 d	1.22	1.09
Bcl-2	4 d	1.24	0.94
	14 d	0.99	0.89
Bcl-xL	4 d	0.89	0.89
	14 d	0.96	1.16
HPRT	4 d	0.87	1.04
	14 d	1.20	0.93
B-actin	4 d	0.88	1.08
	14 d	1.10	1.09
UBQ	4 d	1.17	1.09
	14 d	1.23	1.18

Efficiencies were calculated at, E = 10^ (1/slope)–1. Efficiency values higher than 85% were considered suitable.

Table 6.6. Expression of genes or fold increase between storage treatments

Gene	Broiler blastoderm		6 d old broiler embryo	
	Fold change	Gene status	Fold change	Gene status
Bak	20.10	up-regulated	0.32	down-regulated
Bax	3.48	up-regulated	0.35	down-regulated
Bok	7.08	up-regulated	0.51	down-regulated
Bcl-2	1.19	up-regulated	0.33	down-regulated
Bcl-xL	0.86	down-regulated	0.42	down-regulated

Fold increase=2^-ΔΔCt

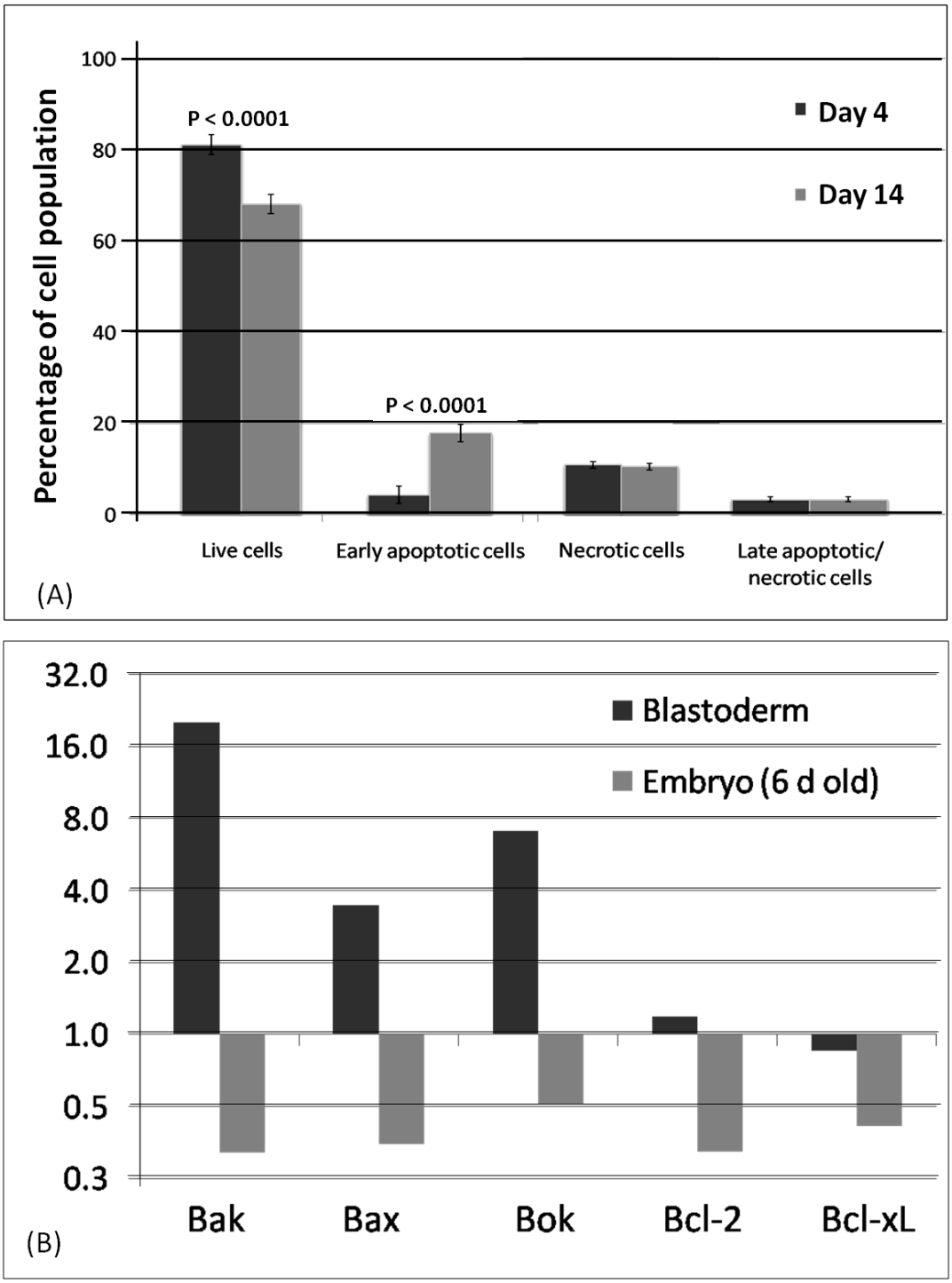

Figure 6.1. Analysis of live, apoptotic and necrotic cells in broiler breeder blastodermal cells population and identification of genes important in the induction of apoptosis in Ross 308 broiler breeder eggs stored for 4 d and 14 d. (A) A flow cytometer-based evaluation of live and dead cells

based on the intensity of two staining fluorescent dyes, Annexin V-FITC and propidium iodide: a) live cells (Annexin V-/ PI-), b) early apoptotic cells (Annexin V+ / PI), c) necrotic cells (Annexin V- / PI+) and d) late apoptotic/necrotic (Annexin V+ / PI+). Percentage of live cells was higher in eggs stored 4 d than 14 d stored eggs ($P < 0.05$; n=3). Percentage of early apoptotic cells was higher in 4 versus14 d stored eggs ($P < 0.0001$). Percentages of necrotic cells and late apoptotic/necrotic cells were not different between egg storage treatments. (B) Gene expression profiling of pro-apoptotic and anti-apoptotic genes. In this figure, we show on a log scale, how different genes were expressed following storage of broiler egg for 4 d and 14 d. Genes that were up-regulated have been shown with positive bars (up) while genes that were down-regulated are shown with negative bars (down). The results indicate that in blastoderms all genes were up-regulated, Bcl-2 did not change but Bcl-xL was down regulated slightly. In broiler embryo from 6 d incubated eggs, all the genes were down regulated.

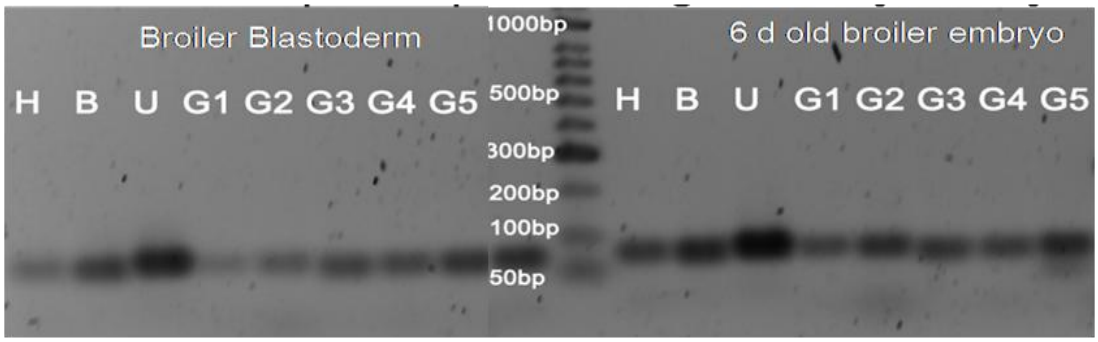

Figure 6.2. Validation of housekeeping and candidate or target genes by Gel electrophoresis of qRT-PCR products on 2% agarose gel. The genes include *H-Hypoxanthine, B-Beta-actin, U-Ubiquitin, G1-Bak, G2-Bax, G3-Bok, G4-Bcl-2, G5-Bcl-xL* and DNA marker in between blastoderm and 6 d embryo samples. All transcript sizes were correspondent to the expected product size of approximately 70bp designed for qRT-PCR analysis.

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CHAPTER 7: GENERAL DISCUSSION

1. OVERVIEW OF RESEARCH FINDINGS

Broiler and turkey embryonic quality is affected by factors such as genetic selection, parent flock, egg storage and other physical factors. Though many researchers have address these factors, an investigation into the physiological, cellular and molecular mechanisms of genetic selection, parent flock age and egg storage are limited. In order to investigate what mechanisms may be involved in determining embryonic survival both at the genetic (strain), epigenetic (flock age) and or environmental (egg storage) levels, a gas calorimetry system was first validated in this thesis to determine the accuracy at which it could be used to monitor gaseous O_2 consumption and CO_2 production during embryonic development (Chapter 3). These measurements are necessary factors that can be used to investigate overheating of embryos during incubation. The study showed that the RQ value (0.64) of the gas calorimetry system used in this thesis was similar to the theoretical RQ value of butane (0.62). The study proved that the calorimetry system was suitable for measurement of embryos gas exchange and metabolic rates. Subsequently the calorimetry system was used to investigate the impact of genetic strain and flock age on turkey embryonic metabolism (Chapter 4) and later effect of egg storage duration on embryonic metabolism (Chapter 6). Both studies have highlighted some of the reasons for overheating of embryos during the later periods of incubation. While there is now evidence that turkey embryos from different parent flock ages metabolize at different rates which could be due to differing respiratory

gas exchange rates, in broiler breeders of the same strain, metabolic output and embryonic body weights decreased following egg storage up to 14 d compared to 4 d stored eggs. In addition, the study on turkey metabolism appears to discover a relationship between embryonic heat production and embryonic mortality during the course of incubation. Importantly, the study has shown that the impact of incubating turkey eggs from different genetic strains is not very crucial when compared to parent flock ages.

Some novel techniques of isolating and separating chicken blastoderms into single cells were also developed in this thesis. Though there are similarly good techniques also used and reported in past studies (Petitte et al., 1990; Bakst and Akuffo, 1999), the applications of their techniques were not repeatable in the case of the current instance. Since the current techniques have been applicable in both layer and broiler blastoderm dissociation procedures, they appear to be reliable for measuring O_2 consumption and CO_2 production. However, the techniques are yet to be applied to dissociate turkey blastoderms. In this thesis particular attention was given to the viability of the individual cells, which may not have been the case in previous studies which required only a few live cells to inject as donor cells during chimera embryo formation. These studies did not also account for cells dying by complex mechanisms such as apoptosis and necrosis which occur through some elaborate procedures compared to typical trypan blue exclusion test. The technique has further been used to evaluate the rate of cell death by apoptosis (programmed cell death) and necrosis (injurious cell death) in stored eggs (Chapters 5 and 6). Through this study it has been demonstrated that storing eggs for prolonged periods

of time decreases the viability of an embryo and could lead to increased embryonic mortality or reduced growth during incubation. The importance of this study will be very beneficial in the future because it has addressed some important questions that were raised more than two decades ago; if cell death is responsible for lowered embryo viability or hatchability; and if the two main mechanisms of cell death (apoptosis and necrosis) are involved in lowered productivity (Fasenko et al., 1992; Bakst and Akuffo, 1999). In addition, the molecular mechanisms were further investigated to confirm if cell death and lower embryo development in stored eggs were induce through altered expression of genes during egg storage and at some time point during incubation (Chapter 6).

The first of the studies in the thesis showed that both O_2 and CO_2 analyzers used in the study were measuring gas exchange accurately (Chapter 3). Generally, results from this thesis showed that embryos from older flock ages produce more heat (Chapter 4). However, to gain one gram of body weight, embryos from young flocks had to produce more heat at 4, 5, 6, 8, and 13 d of incubation when compared to embryos from older parent flock ages (Chapter 4; Figure 4.9). This could imply that, embryos from young parent flock ages were metabolizing at a much faster rate. It is not clear whether these embryos were incubated lower or higher temperatures than normal temperatures resulting in this physiological shift. However, further research has been proposed to investigate how much the temperature should be raised or manipulated in order to determine the exact temperature at which these eggs should be incubated to optimize embryo survival and hatchability. In the past 40-50 years it has been common to select for body weight in order to increased

broiler meat yield and breast meat yield. These changes have altered a lot about all breeders. Kranis et al. (2006) has investigated the strength of genetic association between growth and reproduction traits in turkeys selected for body weight, conformation and egg production. Although increased genetic selection increased body weight, increased selection also affected the physiology of the embryos such that embryos from these broilers outgrew their potential to perform respiratory and metabolic activities properly compared to traditional strains (Christensen et al., 1999; 2008). This physiological change has been reported to cause embryos from one group to grow differently from each other.

Apart from the techniques developed to isolate and separate blastoderms into single cells, the percentage of live cells decreased significantly as both layer and broiler breeder eggs were stored for 14 d compared to eggs stored for 4 d (Chapters 5 and 6; figures 5.2 and 6.1A). The percentage of early apoptotic cells in broiler eggs was significantly higher in 14 d compared to 4 d storage treatment. However, the percentage of necrotic cells and late apoptotic/necrotic cells were higher in only layer eggs stored for 14 d compared to those stored for 4 d. The percentages of necrotic and late apoptotic/necrotic cells in broiler eggs were not different from each other. In unstored eggs, there were very low percentages of live cells and very high percentage of necrotic cells on both FACScan and ImageStream data. While this result was unexpected, the cells from unstored eggs appeared relatively larger than cells from 4 and 14 d stored eggs and contained more than one nucleus (Chapter 5; figure 5.3). The results of this research provide new evidence that reduction in hatchability in stored eggs could be genetically directed through apoptosis.

There is evidence that increased early embryonic mortality is higher in eggs obtained from younger parent flock ages which the exact causes are not known or clearly defined (Reidy et al., 1998; Fairchild et al., 2002). However, experience with isolating chicken blastoderms in the experiments conducted in the current thesis showed that most often such eggs have fragile or incompletely developed blastoderms after oviposition, that is have not reached the usual Eyal-Giladi and Kockav 1976 stage X level of development (personal observation). This was more evident in eggs obtained from younger parent flock ages. The storage of these eggs may further increase the fragility of the blastoderms resulting in more cell death. It is therefore not coincidental that there is lowered productivity from eggs collected from young flocks (Reidy et al., 1998). While storage is a key industrial practice, it has to be selectively applied to reduce its impact on productivity. As part of my investigation in this thesis, it was also observed that as egg storage duration increased from 4 d to 14 d embryonic metabolism reduced and so was embryo weight (Chapter 6; figures 6.2 and 6.3). The long term effect of egg storage such as reduced hatchability could be due to altered embryonic metabolism which could be exacerbated by the reduced number of viable embryonic cells in the 14 d stored eggs.

Therefore the last experiment reported in this thesis (Chapter 6) was used to determine which genes were regulated when eggs were stored for 4 and 14 d prior to incubation or after incubation for 6 d. The results showed that in broiler blastoderms, genes that promoted apoptosis were up-regulated in 14 d stored eggs compared to 4 d stored eggs as shown in Table 6.4 or Figure 6.1 (*Bak* = 20.10, *Bax* = 4.48 and *Bok*

= 7.08) and therefore could be the reason why there is increased apoptosis at the cellular level or in blastoderms (Figure 6.1A). However, genes that prevented apoptosis remained unchanged or were slightly down-regulated following egg storage from 4 to 14 d. In both chicken and turkey embryos isolated from 6 d incubated eggs, there were mixed expressions of pro- and anti-apoptotic genes (Chapter 5 and Appendix 4). The result could imply that heat treatment of eggs before incubation may have had some physiological effects on the developing embryo and thereby affected gene expression. However, in turkey embryos, there was clear indication that heat treatment did not completely deactivate the actions of some pro-apoptotic genes (Appendix 4). This divergent expression of genes between the two species may indicate that there are different physiological developments in chicken and turkey embryos during the time of incubation. This statement agrees with the reasons why turkeys have longer incubation time (28 d) compared to chickens (21 d). In addition, the developmental stages of the turkey blastoderms are slightly different from chicken.

1.1. Embryonic development and heat production

Many researchers have studied the influence of incubation conditions on embryonic development and hatchability. Whereas internal incubation temperature has been utilized to control embryonic development and overheating over the years (Lourens et al., 2005), the knowledge on how much heat the embryo contributes to its own development and how this may affect productivity is still limiting (Hamidu et al., 2007). Especially when it comes to modern genetics strains the current

incubation system appears not to favor embryos because the eggs are still incubated in set ups made for strains that used to exist between 20 to 50 years ago. Therefore readjusting incubator setting such as temperature for specific groups of eggs may be a key to improving survival and reducing embryonic mortality during incubation.

About 10 to 20 years ago, the use of single stage incubators was very popular. But as human population increased the demand for high protein and affordable meat such as poultry has increased. As part of the need to increase productivity and profitability, poultry producers and incubator companies invented the multistage incubators which have the capacity to incubate more eggs. In single stage incubation systems, all the eggs enter and exit the incubator at the same door. The eggs are usually of the same age and there is little variation in incubation conditions in the single stage systems. While the quality and uniformity of flocks produced are normally higher, the need to improve productivity and profitability were of high priority to producers and hatchery managers and therefore in the Americas and certain parts of the world almost all hatcheries have replaced their single stage machines with the multistage ones. In this type of incubation, the incubator contains about 6-8 racks in series. The eggs enter the machine at one door and exit the machine at another door opposite the entry point. The eggs are placed such that the first batch of racks contain eggs of younger embryo ages and as incubation progresses and the embryos grow older they are pushed towards the exit door and a rack containing a new batch of eggs is placed at the beginning. By this time the eggs that are close to the exit door may have reached the time of transfer into a hatcher. Although, the main idea behind multistage incubation is to increase

productivity, make more profit, and maximize incubator space, the rational is also to create a heat current that flows through the incubator and warm the younger eggs that have just been placed in the incubator to save energy.

Normally as eggs get older, they tend to produce more heat as a result of increased metabolism. This heat is circulated through the incubator to warm the eggs that have just been placed in the incubator. However, the combined effects of the incubators own heat and the heat of metabolism from older embryos also affect the embryos of the older batch of eggs. As a result of this the embryos of these eggs may become overheated and this results in most late incubation embryo mortality. Even recent observations from the industry have shown some of the effects of excessive heat production on embryonic development in broiler breeders but this has not been reported in turkey embryos. Nevertheless, a typical problem with multistage incubation system is that it is not possible to adjust incubator temperature to favor the older embryos because reducing the temperature for sake of them may also affect embryos of newly incubated eggs through hypothermia.

Recently, in one particular study involving broiler breeder incubation, there were evidence to show that heat production from two modern broiler strains and parent flock ages were different (Hamidu et al., 20017). In the current thesis using two turkey genetic strains: Hybrid and Nicholas, it has been demonstrated that the difference between genetic strains had no effect on embryonic metabolism but maternal flock age was a factor. However, the egg sizes between these two strains were also not different which may indicate that the lack of physical difference between the eggs may have been reflected in the physiological functions of the

embryos. Nevertheless, as maternal flock age was investigated, the results showed that the impact of age on egg sizes and changes in metabolism were significant. Based on the current and previous research (Hamidu et al., 2007) it can be suggested that it is important to incubator eggs based on flock ages (determines egg size) and afford all embryos an equal opportunity to exhibit their full potential. Currently incubator companies are asking producers and hatchery managers to revert back to the use of single stage machines since conditions in this machine can be easily monitored and adjusted to increased embryo survival and hatchability.

1.2. Factors that affect embryonic development and heat production

1.2.1. Embryonic age

Embryonic development proceeds with incubation time and heat production increases progressively during the incubation process. As clearly shown in chapter 4, a few days after incubation, the embryos begin to produce a noticeable amount of heat as a result of increased O_2 consumption and CO_2 production (Figures 4.1 and 4.2). At this stage, embryonic growth increases embryo weight almost linearly. Heat production also follows the same trend. However, after about 4 d of incubation, heat production increases exponentially and reaches a plateau at about 80% of the time of incubation (Rahn et al., 1979; Dietz et al, 1998; Hamidu et al., 2007). But after internal pipping, embryonic heat production increases again but this is higher than the rate at which embryonic growth may be proceeding. This may create a paradox between heat production and embryo growth and if the embryo is not able to externally pip early enough, it may die as resulting of overheating from internal and

external sources of heat, and possibly CO_2 poisoning and/or shortage of O_2. Heat production from the turkey embryos investigated in the current thesis ranged from approximately 1 mW at d 4 of incubation to 350 mW by 28 d of incubation. The differences in the developmental age of embryos from different ages compared to their chronological age may have influenced heat production.

1.2.2. Biological factors

Biological factors such as genetic strain, breed, breeder age, and egg size can affect embryonic heat production during incubation. Larger eggs have a higher potential to increase heat production and overheating than small eggs (O'Dea et al., 2004). Due to both differences in heat production and heat loss, embryos from large eggs tender grow at a faster rate compared to small eggs. Although egg size was not investigated in the current thesis, eggs obtained from different flock ages differed, directly supplying results on flock age that also applied to egg size. The results in chapter 4 again show that the rate of embryonic growth was clearly higher as parent flock age increased. Although there is not much study about the strains of turkey embryos investigated in the current thesis, other studies have reported that embryos of different genetic strains from both broiler and turkey may have different rates of development and heat production (Christensen et al., 2001; Hamidu et al., 2007). O'Dea et al. (2004) reported that differences in heat production at the plateau stage of O_2 consumption was evidently higher in older parent flock ages of the same strain, but generally heat production was different between broiler breeders and layer eggs. A similar observation has also been made in other studies (Janke et al., 2004;

Sato et al., 2006). These differences could be due to different nutrient or energy utilization of embryos from these strains (Reidy et al., 1998). The effect of flock age and genetic strain may also arise indirectly from different porosities or eggshell conductance and O_2 availability to the embryo, because O_2 availability may affect energy utilization and heat production (Kleiber, 1987). Therefore, an important suggestion made in this study was that eggs from different sources irrespective of strain, flock age and breed should be treated or incubated separately. Therefore, establishing actual temperature profiles of these eggs may further enhance the survival of the embryos and increase hatchability.

1.2.3. Physical and environmental factors

Physical factors like egg storage duration can affect embryonic heat production during incubation. In this thesis when eggs from broiler breeders were stored 4 d and 14 d, it was realized that those stored for a short period of time developed at much better rate than the longer stored eggs. The study agrees with previous results where it was shown that as storage duration increased embryonic viability also decreased while embryonic development slowed down (Bakst and Akuffo, 1999, Foulkes, 1990; Bloom et al., 1998; Fasenko et al., 2002). These effects are due to death of embryonic cells by apoptosis and in some cases necrosis during storage (Chapter 5). In this thesis it was determined that some of the consequences of eggs storage included altered gene expression, reduced viable embryonic cell numbers and reduced embryonic metabolism between 4 d compared to 14 d storage treatments. These effects appear to be linked in loop where the

expression of the genes could be the determining factor to the outcome of the other two factors (Chapter 6).

Although, the environment surrounding the egg was not directly investigated in this thesis, environmental factors such as temperature, O_2 and CO_2 concentrations of the incubator and humidity are crucial factors that could allow the genetic potentials of the embryo to be fully expressed. In general embryos consume O_2, produce CO_2, water vapor and heat during metabolism. But factors such as eggshell conductance, air speed, partial pressure of O_2 and CO_2, vapor pressure may limit the rate at which O_2 and CO_2 can be exchanged between the egg and its immediate surroundings (Ar et al., 1974; French, 1997; Piestun et al., 2009).

Temperature in particular needs to be monitored closely to avoid overheating of embryos and ensure proper embryonic physiology. This could help embryos from different egg sizes, breed, strain and flock age to develop at their optimum. French (1997) explained that during early incubation, embryo temperature is slightly lower than incubator temperature because of evaporative cooling. However, from mid-incubation onwards, metabolic heat production from the embryo raises embryo temperature above incubator temperature. The extent of the rise in embryo temperature depends on thermal conductivity of the eggshell, which, in turn, is mainly influenced by the air speed over the egg. In addition, embryo temperatures can differ from incubator temperature because of differences in thermal conductivity of different incubation systems or types and their ability to control temperatures uniformly (Lourens et al., 2005). Though it is difficult to determine embryo temperature until the embryo is sacrificed, it is the best estimate of embryonic heat

production or metabolism than eggshell temperature. However, because of the difficult of measuring metabolism (if the embryo is alive) the eggshell temperatures are frequently used as best estimate of metabolism or embryo temperature (Lourens et al., 2005). Though eggshell temperature was measured in this thesis by attaching small temperature probes to the eggshell, it was not possible to continue measurement of temperature because the probes were knocked out of place when the eggs were placed in transfer position. From the above discussion, it appears that using eggshell measurements may still not be good a method to assess daily embryonic temperature or metabolism.

Oxygen is the gas that drives the metabolic machinery of the embryonic cells in order to execute the complex process of avian embryonic metabolism and development (Rahn et al., 1979). Oxygen consumption can affect embryonic development right from the time of storage and during the time of incubation. Just few studies have actually demonstrated the effects of O_2 on egg during storage. Onagbesan et al. (2007) reported in their review that storing eggs in a high concentration of air was detrimental to hatchability and had a significant influence on egg quality. In the current thesis O_2 consumption of eggs was not assessed at the time of storage. But it is not clear if O_2 consumption by the eggs (or blastoderms) was limited especially in egg stored for prolonged duration which may have affected the respiration of blastodermal cells resulting in the increased production of apoptosis cells (chapters 5 and 6). As discussed eelier, O_2 consumption is as crucial as temperature during incubation to ensure embryo growth compared to embryos that consumed lower O_2 during incubation (Chapter 4). Throughout this thesis it is

evident that selecting eggs from different turkey parent flock ages and storing chicken eggs can greatly determine the quality of embryos of eggs used for incubation. Oxygen consumption especially can affect the outcome of hatchability and embryo mortality although these were not investigated in my thesis. From the results of the current thesis, it is important to sort out turkey eggs before incubation to optimize embryo performance. In addition, eggs should not be stored for excessively longer periods before incubation unless recommendations such as pre-storage heating and eggs sorting by age which have been proven in other studies promise to reduce some of the adverse effects of egg storage such as reduce metabolism, cell viability and altered expression of some cell loss genes.

2. FUTURE DIRECTIONS

Following my earlier study in broiler embryonic metabolism (Hamidu et al., 2007) incubator companies such as Jamesway Incubation Systems have used the recommendations of the study to advise hatchery managers to switch from using multistage incubation to single stage incubation. This provides the ability to control incubation heat resulting from the fast embryonic growth and metabolism in modern avian species. It is believed that the research in turkey embryonic metabolism will also help to further stress the need to totally control the heat production of embryos during incubation to reduce late embryo morality.

Unlike the intensive poultry production areas in the U.S. that have breeder farms in close proximity to one another and to the hatcheries, Canadian poultry farms are more spread out geographically. This is a positive aspect for improving

biosecurity in the Canadian poultry industry but creates additional transportation costs and logistic challenges. Currently, most hatcheries pick up eggs from farm 2 to 3 times per week in order to decrease the negative effects of egg storage on hatchability and at the same time to reduce cost. While previous research has clearly outlined the negative effects of egg storage on embryo survival (Fasenko et al., 2001a and b), there has been no research conducted to identify the biological reasons why more embryos die after eggs have undergone long-term storage. These answers can only be obtained by using basic research techniques, some of which have been developed in this thesis.

The cell viability research in my thesis will be relevant to the industry because it provides the foundation for future studies aiming to identify genetic strains that can withstand long-term egg storage. In addition, the information provided through this research will be used to design future experiments on egg storage that will promote embryonic cell survival. One of the most valuable aspects of this research is that the techniques developed or refined will provide quick and reliable methods to evaluate the effects of other factors such as genetic selection and parent flock age and their influence on embryonic metabolism, cell viability and gene expression. Through the use of basic scientific techniques from different disciplines such as embryology, immunology (how embryos combat death of embryonic cells), and molecular biology this thesis has the potential to answer basic questions about the physiological effects of egg storage on poultry embryos that to date have remained unanswered. Now that the effects of genetic strain, flock age and cold storage of eggs on embryonic metabolism, cell viability and gene expression

have been understood, hatching egg producers and hatchery personnel can decide on the optimum time to store eggs before incubation or manage incubator conditions to ensure optimum hatchability.

The main recommendation from this thesis is not to store eggs beyond the short-term window if possible (4-7 d) as identified from previous studies (Fasenko et al., 2001b). Fasenko et al. (2001b) showed that storing egg for 4 d versus. 14 d could decrease hatchability of hatching eggs from 90% to 71% and also increase embryonic mortality from 8% to 21%. These numbers identify an area of economic inefficiencies in the entire boiler production chain when eggs are stored up to 14 d prior to incubation. The current research can potentially be used to close this space as to why eggs stored for 14 d have lower performance if the recommendations in this thesis can be applied fully. This research may be especially applicable in turkeys because even under optimum conditions, hatchability of turkey eggs is lower than that of broiler breeder eggs. However, it is important that the cellular and molecular aspects of this thesis are further investigated in turkeys in order to make the turkey production industry as efficient as the broiler production system. Ultimately, this thesis will contribute to the development of sustainable poultry production in Canada and has provided knowledge that supports the production of high quality poultry products that meet consumer demand.

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APPENDICES

Appendix 1: Calibration of embryo metabolism system

1. Detach the two tubes going to the O_2 analyzer just before the T-junction (after the flow meters). This leaves the O_2 analyzer drawing air from the environment. But has small dryer to prevent moisture into the analyzers.
2. Use a 'Y' coupler right beside the CO_2 analyzer to connect (1) the sample air tube from the CO_2 analyzer, (2) the tube going into the sample flow pump and (3) the tube running from the incubator and going into the reference pumps.
3. Connect the nitrogen gas tank to the inlet of the CO_2 analyzer.
4. Open the gas tank and wait till the CO_2 reading on the CO_2 analyzer drops close to zero.
5. Adjust the zero knob of the CO_2 analyzer to 0.5 - 0.8 ppm which will be about - 0.2 to 0.5 on the computer screen.
6. Remove tube from the nitrogen cylinder and connect it to the span gas (21.07% O_2, 2913ppm CO_2 and balanced by N_2).
7. Wait till the CO_2 reaches a peak close to 2913 ppm, probably waiting for 1-2 min. Record the reading from the CO_2 and adjust the span knob to bring the reading on the CO_2 analyzer to 2913 ppm (equal to the CO_2 concentration in the span gas tank).
8. After running the gas through the system for about 5 min, connect the sample and reference tubes just before the 300 ml/min flow meters to the T-

intersections close to the small driers just before the O_2 analyzer (These tubes were detached at step 1).

9. Continue flow of the span gas until the sample and the reference O2 concentration on the screen are stable.
10. To make it easier, open small graphs for the sample and the reference air flow and use them see when the lines are linear or have stabilized. Record the reading kPa.
11. Adjust the O2 analyzer sample and reference knob separately to

    Current atmospheric pressure reading * span gas O_2 concentration

    e.i. = ATM * (21.07/100)
12. Wait till the graph is again straight or stable and close the gas tap.
13. Disconnect and connect all tubes back to their positions.
14. Watch for the possibility of connecting reference and sample tubes to the wrong pump.
15. Turn off the CO_2 analyzer after connecting the tubes and change the dryer in the reference side of the CO_2 analyzer if the need be (every 4 d). Watch out for 2 different dryers and their positions and their orientations when attached to the analyzer. Remember to turn on the analyzer when finished.
16. Take off wet portions of Magnesium perchlorate dryer (just before the sample and reference pumps) and add new one each day. This must be done when both pumps are switched off. And remember to switched them back on after changing or adding dryers (can easily be forgotten).
17. Change the humidifier water tank each day or fill the tank to full.

18. Check that the system pressure is at – 80 psi each day and adjust each day.
19. Check to make sure that the humidifier light is on amber and not on red to be sure that it is working. To adjust humidifier up or down, open incubator and adjust the small meter at the bottom of the incubator.
20. Record humidity and incubator temperature on the small meter in front of the incubator.
21. Adjust chamber flow meters to 100 (during first 7 days of incubation) and then 300 ml/min for the rest of incubation. The flow meters have been calibrated to 100 or 300 with green markings on the flow meters (located at the front-top of the incubator).
22. Adjust the differential pressure (on the screen) to close to zero (-0.2 to +0.2) with the needle valve (just before the inlet of O_2 analyzer).
23. Double check to make sure that all gas tabs are closed and everything is in place before leaving.
24. Record the time everything was done and finish.

Appendix 2: RNA extraction protocol used in broiler blastoderms and embryos

RNA extraction was carried out in Precellys lysing kits. The kit comprise of 0.2 ml microtubes prefilled with beads. The bead filled tubes are used in a Precellys lysing kits (Cayman Chemical, Michigan) that has to remain very cold at all times of its operation.

Extraction procedure

1. Prepare dry ice in the cooling machine. Turn on the air valve, cooling machine and temperature detector about 10 min until the temperature is about 2.8^{o}C.
2. Weight around 100 mg of tissue; put it into the tube with beads (CK-14). Leave the tubes with tissue on dry ice.
3. Add 1 ml of TRIzol to each tube.
4. Load sample unto the lysing machine, cover it and homogenize samples at speed of 5500 rpm, 2 X 30 s with 10 s in between homogenizing.
5. Incubate samples at RT for 20 min.
6. Centrifuge samples at 12,000 g, at 4^{o}C for 10 min. Pour supernatant to a new pre-labeled 1.5 ml tube. Add 200 µl of chloroform. Vortex, incubate at RT for 2-3 min.
7. Centrifuge at 12,000 g, 4^{o}C, for 15 min. Transfer clear supernatant to a new pre-labeled 1.5 ml tube. (At this point only the top clear layer should be carefully taken and avoid too much generosity).
8. Add 250 µl of IPA and 250 µl of a high salt solution (1.2 M NaAc, 0.8 M NaCl). Vortex briefly and incubate at RT for 15 min.
9. Centrifuge at 12,000 g for 10 min. Pour off supernatant and invert the tube to drain.
10. Wash pellet with 1 ml of 75% ethanol. Centrifuge at 8,300 rpm (~5300 g) for 3 min. Pour off supernatant.
11. Solubilize in 100 ul of RNase free water or DEPC water.

12. Check the quality and quantity of total RNA on a Nanodrop spectrophotometer (ND-1000).

Appendix 3: Reverse transcription of RNA sample

DNase treatment of RNA samples

1. Before transcription add 8.0 µl of RNA (100 ηg/µl) and 1.0 µl of DNase (1U/µl) together and incubated at RT for 15 min.
2. Then add 1.0 µl of 2.0 mM EDTA and incubated at 65^{o}C for 10 min, followed by a direct snap chill (4^{o}C) on a PCR machine

Transcription

1. Take 10 µl of the DNase treated RNA and add 1.0 µl oligo dT (0.5 µg/µl), 1.0 ul each of all dNTPs (20 mM) and 0.5 µl of RNase free water in a PCR tube.
2. Heat the mixture to 65^{o}C followed by snap-chill at 4^{o}C.
3. During chilling, add 4.0 µl of 5X first strand buffer, 0.5 µl RNase-out and 2.0 µl DDT (0.1 mM) to the sample in the PCR tubes.
4. Heat the total sample to 42^{o}C for 2 min and add 1.0 µl of superscript II enzyme to the reaction mix.
5. Continue the reaction at 42^{o}C for 50 min and then 70^{o}C for 15 min.
6. After incubation, store the first strand cDNA at -20^{o}C until needed for quantitative RT-PCR analysis.

Appendix 4: Effects of turkey egg storage duration on expression of apoptosis genes

Introduction

Since apoptosis has in the past been speculated in turkey during periods of egg storage some of the genes that orchestrate this mechanism were also investigated on to help raise more interest and awareness in turkey research at the cellular and molecular levels (Bakst and Akuffo, 1999). Over the years investigation in turkey gene expression has been limited due to lack of research or interest turkey molecular studies. While such studies may even be ongoing, an application to solve interesting industrial probrems has not been extensive. Though about 90% of the turkey genome has been sequence, there is a potential to use the chicken genome to reachthe remaining 10% (Dalloul et al., 2010; Richards and Poch. 2003; Hillier et al., 2004). In the short term, completely developing the turkey genome sequence or as has been completed (90%) will provide scientists with knowledge of specific genes that are important in meat yield and quality, health and disease resistance, fertility, and reproduction without necessarily making reference to chicken all the time (http://esciencenews.com). While it will be important to also investigate gene expression coupled with cellular cell death at the blastoderm level, the blastoderm dissociation techniques developed in this thesis to isolate chicken blastoderms has not been validated in turkey blastoderms. This current study was therefore included because it could provide the basis for further cell viability studies in turkeys.

Earlier studies have reported that prolonged storage of turkey eggs reduces viable embryonic cell numbers and that apoptosis and necrosis could account for

reduced performance when the eggs are eventually harvested (Bakst and Akuffo, 1999). This study has the potential to explain some of the fundamental reasons for reduced hatchability as egg storage duration increases in turkey eggs which may leave a permanent effect on the embryo when the eggs are incubated. The aim of this study was to establish the effects of egg storage duration on expression of selected apoptosis genes during incubation.

Materials and methods

In this study, turkey embryos were obtained directly from Hybrid turkeys, a division of Hendrix Genetics Ltd (650 Riverbend drive, Kitchener, ON). The embryos had previously been isolated from turkey eggs stored for 4 d and 14 d under standard hatchery conditions (approximately 18^{o}C and 80% RH). The eggs were then incubated under standard commercial incubation settings (37^{o}C and 56-58% RH) for 6 d. At 6 d of incubation all embryos were removed under sterilized conditions, placed in 1.5 ml microcentrifuged tubes and snap frozen in liquid nitrogen. The samples were placed on dry ice and shipped to the Department of Agricultural, Food and Nutritional Science, University of Alberta. Upon arrival the embryos were quickly transferred to -80^{o}C until RNA was extracted.

RNA extraction and qRT-PCR analysis

A total of 5 embryos from each storage treatment (4 d or 14 d storage) were pooled together and homogenized in liquid nitrogen. A specimen of 250 to 300mg of embryo mass was placed in 14 ml pre-chilled falcon tubes. Total RNA extraction was carried out on the embryo samples using TRIzol (Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008, USA) with a traditional stand alone homogenizer (Appendix 5). All other procedure of transcription, qRT-PCR procedures as well as the process of choosing the housekeeping gene was followed as described in Chapter II (Tables 1 and 2).

Results and discussion

Although the current study did not investigate the deleterious effects of storage on turkey blastoderms at the cellular and the genetic level, results from this study shows that when turkey eggs were stored for 4 d versus 14 d and further incubated for 6 d, there were differential expression of important genes that link embryonic survival and death (Table 3). The results show that as storage duration increased there was mixed expression between both pro-apoptotic (*Bak*, *Bax*, *Bok*) and anti-apoptotic (*Bcl-2, Bcl-xL*) genes. Apart from *Bak* which was down regulated as a pro-apoptotic gene, both Bax (1.7 folds) and *Bok* (2.2 folds) were up regulated. This could indicate that the expression of the two genes is more important than *Bak* to induce apoptosis in turkey embryos as egg storage duration increases. Conversely, *Bcl-2* and *Bcl-xL* expression increased (1.7 and 1.5 fold respectively) as storage duration increased from 4 d to 14 d. While the expression may indicate over protection of embryonic cells to death by apoptosis, (KEGG, 1995-2010), *Bcl-2* and

Bcl-xL have different mechanisms of inducing cell death which may not necessarily means they have to be down regulated.

At the mitochondrial pathway (intrinsic), apoptosis induction results from the broken protective shield from *Bcl-2* and *Bcl-xL* on the surface of the mitochondria. However, under the *p53* signaling pathway of apoptosis, an increased expression of the pro-apoptotic genes particularly *Bax* still cause apoptosis to proceed even though *Bcl-2* and *Bcl-xL* may be highly expressed. In such a case, the cells do not divide into apoptotic bodies but remain as cancer cells (Shen and White, 2001; KEGG, 1995-2010). In other words, although normally responsible for the production of large amounts of antibodies to ensure cell survival, over expressed of *Bcl-2* prevents apoptosis of damaged cell under the *p53* signaling pathway; so damaged cells survive and could progress to a cancerous growth (KEGG, 1995-2010). *Btg2* was down regulation as egg storage duration increased from 4 d to 14 d. Therefore it has no effect in causing apoptosis. The study also shows that although primers used over the turkey DNA were obtained from chicken there were no primer dimmers from qRT-PCR dissociation curves and all the genes were also confirmed to correspond to the appropriate product size expected (Figure 1).

Summary and conclusion

Although this is a preliminary study, it shows that the potential of using existing chicken gene sequences to begin an intensive study in turkey molecular studies is very high. In addition, increased expression of apoptosis promoter gene shows that the effects of egg storage on embryonic development and survival does

not cease even when it is incubated. Therefore eggs should be treated well to avoid any carry over effects from cold storage which could affect the entire broiler production chain.

Table 1. Selecting housekeeping genes base in statistics and coefficient of variation

Gene	Treatment	Av. Ct	P-value	StdDev	CV	Av. CV
HPRT	4 d	25.28	0.0684	0.0631	0.0025	0.0021
	14 d	25.41		0.0457	0.0018	
B-actin	4 d	16.89	0.1529	0.2220	0.0131	0.0073
	14 d	16.78		0.0247	0.0015	
UBQ	4 d	17.65	0.0007	0.0804	0.0046	0.0026
	14 d	17.12		0.0113	0.0007	

Table 2. Real time PCR amplification efficiency for 6 d old turkey embryos

Gene	Efficiency
BAK	1.14
	1.08
BAX	1.13
	0.97
BOK	1.15
	1.06
BCL2	1.14
	0.92
BCLXL	0.88
	1.26
BTG2	1.26
	1.16
HPRT	0.93
	0.99
B-actin	0.89
	0.92
UBQ	0.91
	0.87

Table 3. Expression of genes or fold increase between 14 d and 4 d treatment

Gene	Fold change	Gene status
Bak	0.47	down-regulated
Bax	2.15	up-regulated
Bok	1.74	up-regulated
Bcl-2	1.68	up-regulated
Bcl-xL	1.54	up-regulated
Btg2	0.67	down-regulated

Fold increase=2^-ΔΔCt

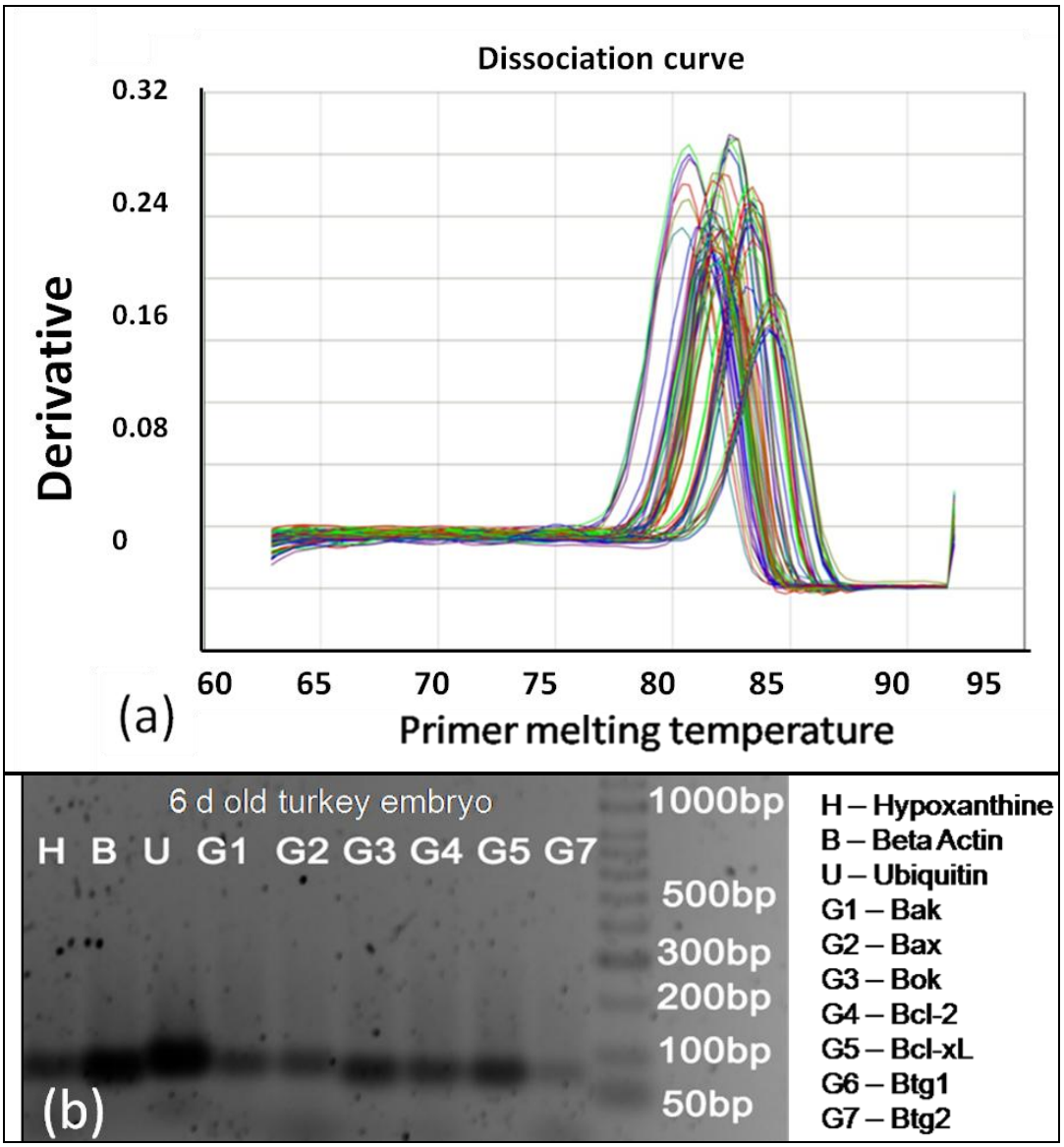

Figure 1. (a) Representative samples of amplification and dissociation curves from qRT-PCR reaction showing primer sets with single peaks. The single peaks show that even though primers were designed from chicken sequences they were still suitable for use in determining gene expression in turkey DNA. (b) Gel electrophoresis of qRT-PCR products. All transcript sizes were consistent with the product size expected from the primers used to amplify the genes as shown in chapter II.

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Appendix 5: Traditional RNA extraction procedure

Probe sanitization

1. Prior to RNA extraction, the homogenizer probe was cleaned with RNaseZap (Applied Biosystems, Ambion 2130 Woodward St. Austin, TX, USA) and wiped off with Kleenex.
2. Probe was further washed with 0.1N NaOH once, three times in MQ H_2O and then once 100% ethanol and let it dry.

Tissue homogenization

1. A total of 5 embryos from each storage treatment (4 d or 14 d storage) were pooled together homogenized in liquid nitrogen in ceramic homogenizer bowl.
2. A specimen of 250 to 300 g of embryo mass was placed in 14 ml pre-chilled falcon tubes.
3. Total RNA extraction was carried out on the embryo samples using TRIzol (Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008, USA).
4. The embryo samples were suspended in 5 ml of Trizol, vortexed, and then homogenized with the probe on ice for 4 times (each for 6 sec and 10 sec in between homogenization).
5. The homogenate was removed and incubated for 20 min at room temperature (RT) to permit complete dissociation of nucleoprotein complexes.

RNA extraction

1. The embryo suspension was centrifuged at 12,000 g for 10 min.

2. The supernatant was transferred into another 14 ml tube followed by addition of 1 ml of chloroform. The sample was vigorously vortexed for 15 seconds and allowed to stay in RT up to 3 min.
3. Suspension was centrifuged at 12,000 g for 15 min.
4. Following centrifugation, the solution separates into two phases: a lower red phenol chloroform phase and a colorless upper aqueous phase. The RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the interface and organic phase. The volume of the aqueous phase is about 60% of the volume of TRIzol used for homogenization.
5. The aqueous phase was transfer into a fresh 14 ml tube and mix with 1.25 ml isopropanol and 1.25 ml high salt solution (1.2M NaAc, 0.8M NaCl) and vortexed briefly.
6. The sample was stored at RT for 15 min and centrifuge at 12,000 g for 10 min.
7. After centrifugation, the RNA formed a white visible precipitation at the bottom of the tube.
8. The supernatant was removed and the RNA pellet washed once with 75% ethanol and subsequently centrifuged at 8,300 rpm (7,400 g) for 3 min. The supernatant was poured off and the pellets allowed to air dried in RT.
9. After air drying the RNA pellet was dissolved in 300 µl RNase free water.
10. The RNA solutions under each treatment (4 d or 14 d) were pooled together and mixed with 0.1 X volume 3M sodium acetate (~30 µl), and 2.5 X volume of 100% ethanol (~330 µl) of the amount of RNA solubilised.

11. The RNA solution was incubated at –20^{o}C overnight to increase precipitation.

12. After incubation, the RNA solution was centrifuged at 12,000 g for 30 min and the supernatant removed. The RNA pellet was again solubilised in 300 µl RNase free water.

13. The quality as well as the quantity of total RNA isolated was determined on Nanodrop spectrophotometer (ND-1000). Unless otherwise stated all centrifugation was carried out at 4^{o}C.