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The chicken embryo and its micro environment during egg storage and early incubation

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When egg storage periods are prolonged (>7 days), hatchability and chick quality declines. The reason for this decline has been investigated, but is still not completely understood. At oviposition the developmental stage of the chicken embryo varies and so do the total number of viable cells. During storage, changes can occur in the embryo. Embryo viability at the end of storage seems to be dependent on the number of viable cells and the developmental stage of the embryo at oviposition. When the hypoblast is completely formed (during the quiescent developmental stage), the embryo seems to be more able to endure prolonged storage periods than embryos that are less or more advanced. During storage, changes also occur in egg characteristics such as albumen viscosity, albumen pH and yolk pH. There appears to be an interaction between albumen pH and embryo viability during early incubation and perhaps also during storage. An albumen pH of 8.2 seems to be optimal for embryo development. Albumen pH may influence embryo viability, but embryo viability may in turn, affect albumen pH. It has been hypothesised that an embryo in which the hypoblast is completely formed is better able to provide an effective barrier between the internal embryo and the exterior (yolk and albumen) and/or is better able to produce sufficient amount of carbon dioxide, which will reduce the pH level in the micro environment of the embryo to the optimal pH of 8.2. It appears that, to maintain hatchability and chick quality after prolonged storage periods, embryonic development should be advanced to the stage in which the hypoblast is completely formed or the atmosphere during storage and early incubation should be altered in such a way that albumen pH is maintained at the optimal level of 8.2.

Keywords: embryo viability; albumen pH; egg storage; pre-warming duration; early incubation

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Introduction

After eggs are laid at the breeder farm, the eggs are collected from the nests, stored in a cool storage room, transported to the hatchery where they are stored once more. After storage the eggs are disinfected, pre-warmed and then set for incubation. Due to variable market demands for day-old chicks in the poultry industry, and maximum hatchery capacity, the total length of egg storage can vary between a few days and several weeks. Egg storage prior to incubation has been reported to have both detrimental as well as beneficial effects (Brake *et al.,* 1993). When eggs are set on the day of oviposition, hatchability declines compared to eggs stored for 4 days (Asmundson and MacIlraith, 1948). Benton and Brake (1996) hypothesised that this is caused by high albumen viscosity in fresh eggs, which impedes oxygen transport to the embryo. Storage periods longer than 7 days under standard conditions (10-20°C and 50-80% RH) cause a delay in hatch time and a decline in hatchability (Becker, 1964; Mather and Laughlin, 1976) and chick quality (Tona *et al.,* 2003). When eggs are set after prolonged storage periods (> 7 days) embryo viability is reduced. To understand the negative effects of prolonged storage periods on embryo viability, two important questions need to be answered:

1) Which factors affect embryo viability during storage and early incubation and
2) How can we prevent the reduction in embryo viability

In order to answer these points, the aim of this review is to identify the changes in the embryo and egg characteristics (micro environment of the embryo) during storage and early incubation. Firstly, the factors that influence embryonic development at oviposition will be described. Secondly, changes in the embryo during storage will be discussed, followed by alterations in the egg characteristics and the relationship between the embryo and the egg characteristics during storage and early incubation.

Information regarding this topic is derived from relatively old literature, therefore embryo characteristics and egg characteristics may have changed in recent decades, due to genetics and management improvement. However the influencing factors remain the same, and the relationship between the embryo and egg characteristics is still important.

Pre-storage embryonic development

MORPHOLOGY OF THE CHICKEN EMBRYO AT OVIPOSITION

Development of the avian embryo begins immediately after fertilization in the infundibulum and continues as egg components are deposited over the next 25 - 26 hours. Most embryonic development occurs while the egg is in the shell gland. This is possible as the body temperature of the hen (41.5°C; Whittow, 1986) is higher than the required temperature for embryonic development. The first 10 - 11 hours in the shell gland, the cytoplasmic mass of the germinal disc cleaves rapidly (Eyal-Giladi, 1991; stages I to VI according to Eyal-Giladi and Kochav, 1976). At the end of the cleavage period the germinal disc is known as the blastodisc (Eyal-Giladi, 1984). Between the 12^{th} and 20^{th} h in the shell gland, two distinct regions are formed: the area pellucida, from which the embryo will develop, and the surrounding area opaca, that gives rise to the extra embryonic ectoderm (Eyal-Giladi and Kochav, 1976). The area opaca and area pellucida are formed by morphogenetic cell movements (stages VII to X according to Eyal-Giladi and Kochav, 1976). The area pellucida consists of 1 or 2 cell layers and the

area opaca consists of 4 to 6 cell layers (Jansonius *et al.*, 1976). When the two distinct regions are formed, the blastodisc becomes a blastoderm.

In avian species there is a considerable variability in embryonic development at oviposition within genetic strains as well as within hens that vary due to the position of an egg in a sequence (Bakst and Akuffo, 1999). According to Eyal-Giladi and Kochav (1976) the most common stage of embryonic development in chicken at oviposition is stage 'X.' More recent research of Fasenko *et al.* (1992a, b) confirmed this and reported that when the developmental stage of the embryo at oviposition was not at stage X, embryos were mostly less developed (Fasenko *et al.,* 1992b; 2001a). In older literature it can be difficult to establish the stage of embryonic development that is being referred to, because the terminology used to describe development stages was not standardized at that time. According to Kosin and Arora (1966), the term "advanced gastrula stage" is the developmental stage of the embryo in which the blastoderm has a distinct area opaca and pellucida (≥ stage X). Blastoderms which do not consist of two distinct regions at oviposition are at an early gastrula stage or pre-gastrula stage.

Several authors have reported that the developmental stage of the embryo at oviposition is related to hatchability (Hays and Nicolaides, 1934; Coleman and Siegel, 1966; Kosin and Arora, 1966; Steinke, 1972). Embryos in a pre-gastrula stage at oviposition appear to give high embryonic mortality during storage.

Coleman and Siegel (1966) compared embryonic development at oviposition of a high and a low weight genetic line. They found that the embryos from the high weight line were less advanced and more sensitive for prolonged storage periods than the embryos from the low weight line. Hays and Nicolaides (1934) and Steinke (1972) divided hens in three groups according to their hatchability and investigated the developmental stage of the embryo in eggs at oviposition. The data showed that pre-gastrula stages at oviposition were common in eggs from hens with hatchability lower than 55%, while eggs from hens with moderate to very good hatchability contained embryos at an advanced gastrula stage (*Table 1*). It seems that developmental stage of the embryo and hatchability are positively associated, but other factors such as genetic selection, or differences in optimal incubation conditions might influence this relationship as well.

Table 1 The variation in the developmental stages of the chicken embryo at oviposition as related to hatchability (Adapted from Steinke, 1972).

Pre-gastrula stage (< stage X*)	Advanced gastrula stage (≥ stage X*)	Hatchability
(%)	(%)	(%)
30	70	>84
30	70	55-84
62	38	<55

*Developmental stages according to Eyal-Giladi and Kochav (1976)

Factors that influence embryo viability at oviposition

As stated earlier, there is a considerable variability in embryonic development at oviposition in domestic avian species such as: turkey embryos and broiler embryos (Bakst and Akuffo, 1999). Several factors influence the developmental stage of the embryo at oviposition, including egg sequence position, sperm quality, and egg passage rate through the oviduct.

EGG SEQUENCE POSITION

Rate of egg production is determined by the pattern termed the egg laying sequence. The first egg of a sequence is generally laid early in the morning. Because egg formation takes normally longer than 24 hours, eggs laid on subsequent days are laid later each day (Warren and Scott, 1935b). A good broiler breeder hen ovulates (and oviposites) an ovum every 25-26 hours (Eyal-Giladi, 1984). Sequences are terminated by days on which no egg is laid; called a 'pause day'. A pause day is caused by a delay in ovulation of the F1 follicle. Ovulation of the follicle is stimulated by a lutenising hormone (LH) surge which can only be initiated in a 6-10 hour 'open period' per day, and is dependent on progesterone production from the mature follicle. The open period is the only time when the hypothalamus can respond to a progesterone signal. When the F1 follicle is not mature enough during the open period to produce sufficient amounts of progesterone, the LH surge will not reach the peak required for ovulation and consequently ovulation will not occur (Robinson, 2002).

The time between oviposition of the last egg of one sequence and the oviposition of the first egg of the succeeding sequence can be 40 hours or more (Robinson *et al.,* 1991). An ovarian follicle destined to become a first-of-sequence egg resides as the largest follicle on the ovary for about 16 hours longer than the subsequent follicle (Scott and Warren, 1936). It is shown that embryos in first-of-sequence eggs are more highly developed than those from subsequent eggs (average developmental stage: 10.36 and 10.05, respectively; Fasenko *et al.,* 1992a; stage of development according to Eyal-Giladi and Kochav, 1976). The reason why the embryo in the first of sequence egg is more developed than in subsequent eggs is not known. It may be that the first egg of a sequence remains longer in the shell gland than subsequent eggs (Berg, 1945) or that aging of the follicle in the ovary results in a faster onset of development due to intra-follicular processes. The latter has been seen in bovine cumulus-oocyte-complexes (Wit and Kruip, 2001). According to the results of Steinke (1972), embryonic development at oviposition has a positive relationship with hatchability (see 2.1.). Because the embryos in first of sequence eggs are more advanced at oviposition, it can be assumed that hatchability of these eggs would be better than that of subsequent eggs. However, Fasenko *et al.* (1992a) showed that hatchability of first of sequence eggs was lower than of subsequent eggs (93.5% and 96.6%, respectively). Robinson *et al.* (1991) and Fasenko *et al.* (1992a) speculated that the lower hatchability of embryos from first-of-sequence eggs might be due to pre-ovulatory aging of the oocyte or changes in yolk composition that affect embryonic growth. According to this research, it can be concluded that more advanced embryos at oviposition are not always a guarantee of good hatchability. The decline in hatchability observed in older breeder flocks, might be related to an increase in the incidence of first of sequence eggs when the hen ages. Sequence length normally decreases from the time of peak egg production to the end of the egg production period of the flock.

SPERM QUALITY

At ovulation the ovum is surrounded by the inner perivitelline layer (IPVL). Fertilization of the ovum takes place in the infundibulum of the oviduct (Olsen and Fraps, 1944). Spermatozoa bind to the inner periviteline layer (IPVL) and undergo the acrosome reaction (Bakst and Howarth, 1977). Acrosomal enzymes that hydrolyse the IPVL are released and spermatozoa are able to enter the ovum through holes created by these enzymes (Howarth and Digby, 1973). As the ovum passes through the oviduct, it is covered with the outer perivitelline layers, which trap spermatozoa and prevent further sperm-ovum interactions (Bakst and Howarth, 1977). The number of spermatozoa in the perivitelline layer of the yolk indicates the potential for fertilisation.

Several factors influence the number of spermatozoa in the perivitelline layer (Brillard, 1993). These factors include semen dose (Taneja and Gowe, 1962), the number of suitable spermatozoa deposited (*i.e.* sperm that has the capacity to survive the selection procedure in the hen's oviduct and to reach the sperm storage tubules; Brillard, 1993), the duration of sperm storage in the hen's oviduct (Lodge *et al.,* 1971; Brillard and McDaniel, 1986) and age of the hen (Brillard and McDaniel, 1986). Nalbandov and Card (1943) showed that embryos of eggs fertilized by 'stale' sperm (sperm held in the hen for a long period) more often terminated their development prior to hatching than embryos in eggs fertilized by fresh semen. Nalbandov and Card (1943) summarized hatchability data of eggs fertilized by sperm of varying degrees of staleness, and showed that average hatchability was 44.5% between 13 -16 days and 74.1% between 1-12 days after the last mating. Brillard and McDaniel (1986) reported that embryonic mortality during the first week of incubation significantly increased by approximately 4% when the time after artificial insemination was extended from one to two weeks. These results were observed in both young (28 to 31 wks) and old (49 to 52 wks) hens. Embryonic mortality during mid and late incubation was not affected. Fasenko *et al.* (1992a) showed that the developmental stage of the embryo decreased from stage 10.3 to stage 10 when the days after insemination increased from 2 to 8 days.

Based on these results, it seems that factors that negatively influence fertility may negatively influence the developmental stage of the embryo at oviposition and decrease hatchability due to an increase in early embryonic mortality. Mechanisms involved in the fertilization of the ovum will not be further discussed in this review, but have been included as they might influence embryo viability at oviposition.

THE EGG PASSAGE RATE THROUGH THE OVIDUCT

Warren and Scott (1935a) measured the total oviduct length in 19 hens, and found it ranged between 555 and 730 mm. They did not investigate whether the passage rate of eggs varied between the hens and whether it was related to oviduct length. The time an egg spends in the different sections of the oviduct was recorded in two other studies (Warren and Scott, 1935a, b). It was concluded that the differences in interval length between ovulation and oviposition is caused by a variation in the time an egg spends in the shell gland. Berg (1945) showed that the last egg of a sequence stays in the shell gland longer than first and subsequent eggs. This increased time may explain the increased embryonic development in terminal sequence eggs at the time of oviposition (Robinson *et al.,* 1991). The time an egg spends in the shell gland appears to be correlated with the position of an egg in a sequence. It is not known whether the variability in embryonic development at oviposition, caused specifically by sequence position, is significant enough to make a difference in embryo viability and consequently, hatchability.

In conclusion, embryo viability and the likelihood that a viable chick will hatch from an egg seem to be associated with the developmental stage of the embryo at oviposition. The developmental stage of the embryo at oviposition may be influenced by egg sequence position, sperm quality, and the egg passage rate through the oviduct during egg formation. It is likely that these three factors are influenced by the age of the breeder flock.

The chicken embryo during storage

After oviposition the embryo is exposed to different environmental factors which may affect embryo viability and therefore hatchability and chick quality.

PATTERN OF EGG COLLECTION

After oviposition, the developmental stage of the embryo is influenced by the environmental temperature in the nest and the time period between oviposition and egg collection. Fasenko *et al.* (1999) investigated these aspects in eggs from a 41 week old breeder flock and found that embryos of eggs that remained longer (3.5 to 6.5 h) in the nest at an environmental temperature of 28°C were more developed than eggs collected just after oviposition and stored at 18.9 to 20.7°C for 10 hours (stage 11.67 and 10.38, respectively; according to Eyal-Giladi and Kochav, 1976). These results were likely caused by the longer exposure to temperatures that enhanced embryonic development. Hatchability of fertile eggs and the incidence of early embryo mortality were not significantly affected by the length of nest holding. In this study the storage period was 2 to 5 days, which may have been too short to establish a positive relationship between embryonic development and hatchability, as was shown by Fasenko *et al.* (2001a, b).

The above is supported by Meijerhof *et al.* (1994) who did not find any effects of holding eggs from a 37 week old breeder flock at simulated nest temperatures (10°C, 20° C and 30°C) after oviposition. However, hatchability from fertile eggs was reduced by 2.4% when a nest temperature of 30°C was compared with 20°C in breeder flocks aged 59 weeks. They suggested that eggs produced by older birds were more sensitive to high temperatures in the nest box than young flocks. These results show that the viability of the embryo can be affected in the time between oviposition and egg collection. The effect of nest holding temperature seems to be interrelated with different factors such as storage period and breeder flock age.

CHANGES IN THE CHICKEN EMBRYO

It has been shown that prolonged egg storage prior to incubation has a negative effect on hatchability (Becker, 1964; Mather and Laughlin, 1976) and chick quality (Tona *et al.,* 2003). An important question that still remains unanswered is: what happens to the embryo during storage? As stated earlier, one factor that highly influences embryonic development is temperature. Early research suggested that no developmental changes occur in embryos when eggs are stored at temperatures well below normal incubation temperature. This temperature was termed ‘the physiological zero’ and was reported to be either 20-21°C (Edwards, 1902), or 24 to 27°C (Funk and Bellier, 1944).

Arora and Kosin (1968) did not observe any change in the gross morphology of the blastoderms during storage periods up to 21 days when eggs were stored at 7.2, 12.8, and 18.3°C, but observed changes in the cellular activity of the blastoderms. With increased storage period, there was a rise in the number of mitotic and necrotic indexes at all three temperatures, particularly in the 12.8 and 18.3°C temperature groups. The proportion of mitotic cells seemed to accumulate during storage and therefore, they hypothesized that the nuclei were blocked at metaphase. Due to the parallel rise of necrotic nuclei, it was also hypothesized that many of the blocked mitotic nuclei died during storage. At a storage temperature of 7.2°C, the cellular activity in the blastoderm was only marginal and therefore they suggested that a storage temperature of 7.2°C was more suitable for the preservation of viable cells than 12.8 or 18.3°C , when storage is prolonged.

Bakst and Akuffo (1999) investigated the total number of blastoderm cells of turkey embryos at oviposition and after 2, 4 and 14 days of storage at 18°C. The total number of blastoderm cells was 32,000, 21,500, 19,000, and 21,000, respectively. Thus, within the first 48 hours of storage the total number of blastoderm cells decreased by 30%. The decrease in cell numbers could be due to both apoptosis and necrosis. Bloom *et al.* (1998) investigated the percentage of apoptotic cells in chicken embryos and found on average

3.1% apoptotic cells just after oviposition, which increased to 13.9% after 14 days of storage at 12°C.

The ability of an embryo to survive storage may vary between domestic avian species due to differences in embryonic development at oviposition. At oviposition, the developmental stage of the turkey embryo is characterized by the initial appearance of the area pellucida (Gupta and Bakst, 1993), while in the chicken embryo the area pellucida formation is already completed (Eyal-Giladi and Kochav, 1976). It is possible that at oviposition the embryonic developmental stage of the turkey embryo is more sensitive than the chicken embryo. Arora and Kosin (1966) showed that some turkey blastoderms already contained numerous vacuoles in the opaca and pellucida after 1 to 2 days of storage, while in chicken embryos this occurred after 14 days of storage. Previous research quantified the number of blastoderm cells in chicken (Spratt and Haas, 1960; Radatz *et al.,* 1987) and turkey embryos (Bakst and Akuffo, 1999). Turkey blastoderms had much lower number of cells, and so cell death could have a greater impact in this species. Because the total number of blastoderm cells is likely to be variable at oviposition, it may be difficult to predict the severity of the damage caused by cell death during storage.

It can be concluded that, during storage, cell death occurs and cells are probably able to initiate mitosis even when eggs are stored below the physiological zero of 20°C. To halt or reduce this cellular activity in the embryo, eggs should be stored at a temperature around 10°C. In nature, eggs are not stored under tightly controlled conditions and therefore, cell death probably occurs. In wild avian species, the temperature of the eggs is alleviated by short incubation periods when the hen is on the nest to lay the next egg in the clutch. These short incubation periods may advance embryonic development. It can, therefore, be hypothesized that when the total number of cells increases, the overall percentage of cell death decreases and embryo viability improves.

PRE-STORAGE INCUBATION

Several authors have investigated the effect of pre-storage incubation on hatchability and chick quality (Becker and Bearse, 1958; Bowling and Howarth, 1981; Fasenko *et al.,* 2001a, b; Lourens, 2006; Renema *et al.*, 2006). Fasenko *et al.* (2001 b) placed broiler breeder eggs in an incubator at 37.5°C for 0, 6, 12, and 18 hours and then stored them for 4 or 14 days afterwards. Embryonic development advanced significantly with increasing length of the pre-storage incubation period. Hatchability of eggs stored for 4 days was not influenced by pre-storage incubation. Hatchability of eggs stored for 14 days was significantly better after 6 hours of pre-storage incubation than without pre-storage incubation. Lourens (2006) subsequently confirmed a positive effect of pre-storage incubation on hatchability of broiler breeder eggs. In comparison with the control group, the increase in hatchability after 3, 6 and 9 h of pre-storage incubation and a storage period of 14 days was 9.2% ($P<0.05$), 11.8% ($P<0.05$) and 6.4% ($P>0.05$), respectively. Hatchability between the three treatment groups (3, 6, and 9 h) was not significantly different.

Fasenko *et al.* (2001b) suggested that there was an optimal developmental stage of the embryo that is better able to withstand prolonged storage periods. Improvements in hatchability after pre-storage incubation were not simply due to lengthening the total incubation period. The optimal pre-storage incubation treatments that improved hatchability of long term stored eggs, advanced embryos to the developmental stage in which hypoblast formation was complete (stage XII, according to Eyal-Giladi and Kochav, 1976). These embryos were probably more resistant to prolonged storage periods than embryos that did not complete hypoblast formation (< stage XI) or embryos that started to form the primitive streak (> stage XIII).

The formation of the primitive streak is a period of active cellular migration and differentiation of embryonic cells. Fasenko *et al.* (2001b) hypothesised that it was not favourable to store embryos in this developmental stage for prolonged periods, because storage would impede critical cellular and embryonic processes. A storage period of 4 days, however, was not deemed detrimental in embryos that already formed the primitive streak. This data gave rise to the hypothesis that the survival of embryos that have reached this active stage of development (primitive streak formation) was dependent upon the length of storage to which they have been exposed. The beneficial effect of pre-storage incubation is therefore dependent on the interaction between the developmental stage of the embryo at oviposition, the storage period, and the length of the pre-storage incubation period. The results of Fasenko *et al.* (2001b) also showed that hatchability after 6 hours of pre-storage incubation and 14 days of storage was not equal to hatchability after 0 hours of pre-storage incubation and 4 days of storage. It can be suggested, that maintenance of viable cells during storage was better than compensating for cell death, by increasing the developmental stage and the number of cells through pre-storage incubation.

Egg characteristics during storage

During storage, changes occur in egg characteristics. Because these characteristics form the micro environment surrounding the embryo, it is possible that they may affect cell death and/or embryo viability. Changes in the egg characteristics, therefore, merit discussion.

CHANGES IN ALBUMEN PH AND INFLUENCING FACTORS

At oviposition the albumen pH is around 7.6 (Stern, 1991). After oviposition, carbon dioxide is released from the egg. Due to the release of carbon dioxide, the equilibrium of the carbonate-bicarbonate buffer system is thought to be shifted towards production of carbon dioxide. Consequently, albumen pH rises to pH 9 after four days of storage and does not increase much more thereafter (Lapăo *et al.,* 1999). The rise to pH 9 may occur to protect the embryo from microbial contamination. The increase of albumen pH depends predominantly on the buffering capacity of the albumen (Benton and Brake, 1996), but also on temperature (Goodrum *et al.,* 1989), storage time, gaseous environment in the storage room (Walsh *et al.,* 1995), and conductance of the eggshell (Meijerhof, 1994). The buffering capacity of fresh albumen is weakest between pH 7.0 and 9.0 (Benton and Brake, 1996). Between 0 and 4 days of storage the albumen pH is within this range and, therefore, the pH increases quickly.

CHANGES IN ALBUMEN VISCOSITY AND INFLUENCING FACTORS

At oviposition the albumen viscosity is maximal (Silversides and Scott, 2001), and decreases after oviposition. The mechanisms responsible for this decrease are not completely understood, but possible mechanisms involved in albumen thinning have been described in detail by Shenstone (1968) and Burley and Vadehra (1989). They suggest that the loss of carbon dioxide plays an important role in the mechanism of albumen thinning. Factors that affect the viscosity of the albumen by directly or indirectly influencing pH included storage time, storage conditions and age of the breeder flock (Scott and Silversides, 2000). The loss of albumen viscosity is not linear with temperature, but increases progressively with increasing storage temperature. Haugh unit score was found to decline more slowly as storage temperature decreased towards 0°C (Proudfoot, 1962).

To reduce the decline in albumen viscosity, Williams (1992) proposed that storage temperatures should be maintained below 10°C. Preventing a decrease in albumen viscosity during storage might be necessary so that enough ovomucin remains for the developing embryo during incubation (Hurnik *et al.,* 1978). Albumen and chalazae are protein gels, which consists of ovomucin fibres to which water is bound (Fromm, 1966). McNally (1943) showed that the condition of the ovomucin changed when albumen pH changed. At pH between 6.0-6.4 and 8.3-8.5, the ovomucin exists in the gel form, and at higher pH values, it exists as a viscous solution. These protein gels seem to have an optimum pH, at which point the fibres will bind the largest amount of water (Fromm, 1966). According to the results of McNally (1943) this optimum pH level is approximately 8.3-8.5, which was confirmed by McKerley *et al.* (1967). They investigated the change in albumen pH and the deterioration of thick albumen, when consumption eggs (table eggs) were stored at different atmospheres that affected albumen viscosity and albumen pH.

As previously discussed, albumen pH is around 9 after four days of storage at standard storage conditions. Without manipulation of the storage conditions, it is impossible to maintain an albumen pH of 8.2 during prolonged storage periods. Because pH of 8.2 seems to be important in maintaining albumen viscosity two questions arise:

- Whether albumen pH and viscosity are optimal for maintaining embryo viability during storage and for embryonic development during early incubation and
- How the two optimal pH levels of 9 (to prevent microbial contamination) and 8.2 (optimal level for albumen viscosity and perhaps embryo development) are combined in one egg?

WATER LOSS AND INFLUENCING FACTORS

After oviposition the egg starts to lose water to the environment due to the pressure differences between the inside and the outside of the egg. The albumen contains the highest amount of water of all egg components. The albumen loses water to both the environment and the yolk. Due to water movements, the osmolarity of the albumen and yolk changes. Loss of water to the environment is influenced by the environmental temperature, relative humidity, egg storage duration, and age of the breeder flock (Walsh *et al.,* 1995). Initially, water that evaporates through the pores of the avian egg comes from the shell membranes. This is replaced, to some extent, by recruitment of water from the albumen. The amount of water in the shell membranes depends on an equilibrium between the capillary tension of the membranes and the colloid osmotic tension of the albumen. Brake *et al.* (1993) stated that water loss from the albumen may have a negative influence on the viscosity of the albumen, although later Benton and Brake (1996) were not able to find a direct relationship between water loss and albumen pH and Haugh Units.

Meijerhof *et al.* (1994) showed that water loss between egg collections made at 17 days of incubation was not affected by the relative humidity (55 or 75%) during a storage period of 7 days. According to these results, they suggested that the influence of water loss during storage on hatching results is limited, under practical conditions. Although the loss of water during storage is minimal compared with the loss of water during the whole incubation period, it is often advised to minimize water loss during storage (Mayes and Takeballi, 1984; Walsh *et al.,* 1995).

CHANGES IN THE YOLK AND INFLUENCING FACTORS

At oviposition, the yolk has a pH of 6 to 6.3 (Stern, 1991). After oviposition, the pH of the yolk rises slowly to a pH around 6.5 to 6.8 (Shenstone, 1968; Bakst and Holm, 2003). The buffer system of the yolk is not based on bicarbonate, as in the albumen. During

storage the yolk index (ratio of yolk height and width) changes. The vitelline membrane surrounding the yolk becomes weak and the yolk has a tendency to flatten (Fromm, 1966). After oviposition, water moves from the albumen to the yolk, due to differences in osmotic pressure, and this might cause the change in yolk index and the weakening of the vitelline membrane. Fromm (1966), however, suggested that the water content of the yolk does not necessarily affect the yolk index. Fromm (1966) showed that, even with a high water content, the yolk index was high for those eggs in which the albumen was maintained at or below pH 8.0. These results suggest that albumen pH is the most important factor influencing the strength of the vitelline membrane and the yolk index. This is in agreement with earlier findings of Fromm (1964), who hypothesised that the strength of the vitelline membrane was highly dependent on the quality of the chalaziferous layer surrounding the yolk. The chalaziferous layer is a layer of fibres and gel-like substances, and, as stated earlier, the viscosity of a gel is highly dependent on pH. Because the viteline membrane and chalaziferous layer are the boundary between the embryo and the albumen, the quality of the chalaziferous layer and vitelline membrane are important because they play a role in the protection of the embryo during the first few days of incubation when the amnion is not yet formed (Sadler, 1955).

Relationship between the embryo and changes in egg characteristics during storage and early incubation

The embryo on one side is directly in contact with the yolk and, on the other side, touches the inner perivitelline membrane, and is close to the chaliziferous layer of the albumen. Because of this, the yolk and chaliziferous layer form the micro environment of the embryo, which is subsequently exposed to the pH difference between the yolk and chalaziferous layer of the albumen. After four days of storage, when the pH of the chalaziferous layer is 9, there is a difference of pH3 (yolk pH being 6). Benton and Brake (1996) hypothesised that the difference in pH between albumen and yolk is necessary for particular transport functions through the vitelline membrane. However, it is also possible that exposure of the embryo to high albumen pH levels can become detrimental to the embryo when storage is prolonged. Several authors have tried to investigate the optimal micro environment of the embryo during storage (Becker, 1964; Becker *et al.,* 1968; Steinke, 1969; Reinhart and Hurnik, 1982; Walsh *et al.,* 1995; Lapão *et al.,* 1999), but this is still not known.

EMBRYO AND EGG CHARACTERISTICS DURING STORAGE

Albumen pH increases to pH of 9 within four days of storage (Lapão *et al.*, 1999*)*, perhaps to ensure protection against microbial contamination. As stated earlier, pH of 9 does not correspond with the optimal pH level for maintenance of the strength of the vitelline membrane, the yolk index, and albumen viscosity (8-8.2; McNally,1943; Fromm, 1966; McKerley *et al.,* 1967). Although the latter was investigated in table eggs, it agrees with the optimal pH level of the extra cellular space of the embryo after 24 hours of incubation, as found by Gillespie and McHanwell (1987). They investigated the pH in the extra cellular space in isolated chicken embryos in vitro at stages 4-22 somites (normally between 26 and 53 hours of incubation). The measured pH varied between 7.9 and 8.4. Experiments done earlier in their lab showed that fibroblast migration is faster at pH 8.2 than at pH 7.4. It seems, therefore, that the optimum pH for embryonic development during the first few days of incubation is between 7.9 and 8.4. When Gillespie and McHanwell (1987) decreased the pH of the bathing medium of the embryo to 6.8 or increased it to 9.0, the intra-embryonic pH changed in the same

direction, but only by 0.1-0.2 pH units. These results suggested that the ectodermal and endodermal epithelia of the embryo formed an effective barrier between the inside of the embryo and the exterior, and protected the embryo from suboptimal pH levels.

It is not known from which age onward the embryo is able to form such an effective barrier and whether the pH of 7.9-8.4 is also the optimal pH for the embryo during storage and the first few hours of incubation.

Walsh *et al.* (1995) hypothesized that the maintenance of the barrier might cause a depletion of energy reserves, resulting in embryonic death. Radatz *et al.* (1987) showed that oxygen fluxes increased in the posterior region of the stage X blastoderm, which is probably connected to the onset of the expansion of the primary hypoblast. As the number of viable cells increase in this region, cell density increases as does metabolic activity. It can be hypothesised according to these measurements that, when the oxygen consumption of the embryo cells increases, carbon dioxide production from the embryonic cells increases at the same time. Consequently, during storage and early incubation, it may be that a more advanced embryo has a greater number of viable cells and is probably better able to form an effective barrier between the inside of the embryo and the exterior and/or is probably better able to produce a sufficient amount of carbon dioxide than a less advanced embryo. More carbon dioxide production will reduce the pH level in the micro environment of the embryo from a pH of 9 to a pH of approximately 8. In this way the metabolism of the embryo regulates the pH level of the micro environment and the yolk index, strength of the vitelline membrane and albumen height. The albumen viscosity that corresponds with an albumen pH of 8.2 might reflect the oxygen requirements of the developing embryo during early incubation and its protein requirements during the total incubation process (Hurnik *et al.,* 1978). The embryo will maintain its protection against microbial contamination, because the pH of the outside layers of the albumen stays at a pH of 9.

If this hypothesis is correct, the developmental stage of the embryo, the number of viable cells and the pH of its micro environment are the most important factors that affect embryo viability during storage and early incubation. It can be hypothesized that an embryo at oviposition (stage X; according to Eyal-Giladi and Kochav, 1976) is not able to form an effective barrier between the inside of the embryo and the exterior, nor produce sufficient amounts of carbon dioxide to regulate the pH of its own micro environment. In such situations, it is important to alter the storage and early incubation atmosphere to maintain the albumen pH at 8.2.

MANIPULATION OF EGG ENVIRONMENT DURING STORAGE

Many authors have investigated the effect of altering the storage atmosphere during storage on hatchability. The results of these different studies are not consistent and are often difficult to explain. Proudfoot (1964) investigated the effect of packing eggs in plastic bags (Cryovac and Poly. No. 100) supplemented with an unknown amount of carbon dioxide. The use of supplementary carbon dioxide in the plastic bags had a severe depressing effect on hatchability. Hatchability of eggs that were packed in the Cryovac bags and supplemented with carbon dioxide was reduced to zero when the storage period was 14 and 21 days. Packing eggs in a plastic bag without supplementary carbon dioxide was deemed beneficial. In two other studies, Proudfoot (1965; 1972), showed via different tests, that packing eggs in plastic bags supplemented with nitrogen gas had a positive effect on hatchability. Proudfoot (1965) showed that the Cryovac nitrogen packing method tended to maintain the oxygen level at about 4% and stabilised albumen and yolk pH. Albumen pH was maintained at the fresh egg level. Proudfoot (1965), proposed that temporary displacement of oxygen by nitrogen could result in more chemical stability of the egg components during the pre-incubation period. He also

suggested that high carbon dioxide levels had a severe toxic effect on the embryo. Whether this hypothesis is true, is not known. It seems that a prevention of gaseous exchange during egg storage has a positive effect on hatchability and that low oxygen levels during storage is not detrimental for hatchability. Becker *et al.* (1968) investigated the effect of high levels of carbon dioxide in the albumen at the end of the storage period (prior to setting) on hatchability. The idea was to bring the albumen pH back to the level of a fresh laid egg just prior to incubation. Before setting, eggs exposed to different storage periods were placed in a Cryovac plastic bag and filled with carbon dioxide gas for one hour. Due to the carbon dioxide treatment the albumen pH decreased almost to the level of a freshly laid egg (7.937 and 7.907, respectively). There were some differences observed in the hatchability of the fertile eggs between the control and treated eggs, due to different storage periods.

This effect on hatchability was not consistent, and the average hatchability, over all storage periods (from 0 to 21 days), indicated that there was no difference between the controls and the treated eggs (on average 79.1% and 78.8%, respectively). After 24 hours of incubation, the albumen pH of treated and control eggs were almost similar (9.201 and 9.296, respectively). During storage, the albumen pH was comparable for the treated and control eggs. The only difference was seen in the pH levels at the start of the incubation process. Becker *et al.* (1968) therefore suggested that the high level of albumen pH during storage negatively affected embryo viability after a prolonged storage period. It may be possible that the increase in albumen pH during the first 2 days of incubation negatively affected embryonic development.

EMBRYO AND EGG CHARACTERISTICS DURING EARLY INCUBATION

The first part of the incubation process is the developmental phase, which is defined as when the embryo forms from the area pellucida. The following membrane and fluid compartments are formed from the area opaca:yolk sac membrane (formed by the area *vitellina* and area *vasculosa*), amnion (surrounds the embryo by day 4 of incubation), allantois (a sac growing out of the primitive hindgut of the embryo from day 2 of incubation), chorion (forms the chorio-allantois, finished around day 11 of incubation), and sub-embryonic fluid (SEF) (becomes evident around day 2-3 of incubation). These membranes and compartments serve to protect the embryo during development and assist in providing nutrition, excretion, and respiration of the embryo (Nechaeva *et al.,* 2004).

As described previously, the chalaziferous layer and vitelline membrane protect the embryo prior to completion of the amnion development. Sadler (1955) investigated the breakdown of the viteline membrane and chalaziferous layer and the closure of the amnion during early incubation. He suggested that until 24 hours of incubation, the vitelline membrane and the chalaziferous layer supported the yolk and protected the developing embryo. At 72 hours of incubation the vitelline membrane was absent over the area of the embryo, but was still present outside the yolk sac. During this period of incubation, the chalaziferous layer is the only protection that the embryo has from the alkaline albumen. At 96 hours of incubation the chalaziferous layer was absent in the majority of the investigated embryos. The results reported by Sadler (1955) showed that the closure of the amnion can be variable, ranging from less than 72 hours up to 96 hours of incubation. Some very slow developing embryos showed little extra-embryonic development even after 80 hours of incubation. This led to the assumption that the amnion of a number of embryos may not be closed at the time of disappearance of the vitelline membrane and chalaziferous layer. The variability in closure of the amnion might be caused by a delay in embryonic development caused by prolonged storage periods. This could lengthen the time that the embryo is exposed to

the alkaline albumen, because amnionic development is slower after prolonged storage periods. Consequently, the embryo might be damaged or even die.

With the above results in mind, it seems to be important to prevent a delay in the onset of embryonic development at the start of the incubation process. Published work has shown that delays occur in embryonic development after prolonged storage periods (Kaufman, 1938; Steinke, 1972). Kaufman (1938) found, according to the developmental stage of the embryo, that embryos from eggs stored between 24 and 34 days started to develop 24 hours later than embryos from fresh eggs, once proper incubation temperatures were provided. Embryonic mortality during the first week of incubation was also significantly higher in stored eggs than in fresh eggs.

It may be that the delay in embryonic development after prolonged storage periods could be reduced if eggs are pre-warmed fast from storage temperature to the optimal eggshell temperature of 100°F (Lourens, *et al.,* 2005) at the start of the incubation process. Little information in the literature is available regarding the effect of the rate of pre-warming at the start of the incubation process on hatchability and chick quality. Meijerhof *et al.* (1994) investigated pre-setting temperatures on hatchability of fertile eggs produced by two breeder flocks at two ages (37 and 58 weeks). Before setting, the eggs were treated in two ways: 16 hours at 20°C or 16 hours at 27°C. No difference was found in hatchability of fertile eggs of the young breeder flock, but hatchability of fertile eggs of the old breeder flock was significantly higher in the pre-warming temperature treatment of 20°C (89%) compared to 27°C (85.1%). As stated earlier, embryos of older breeder flocks seem to be more sensitive to temperatures above 20°C (Meijerhof *et al.,* 1994). Abnormal development usually occurs in the temperature range of 27°C to about 35°C (Wilson, 1991) and this might explain the negative effect of a pre-warming period of 16 hours at a temperature of 27°C in the older breeder flock. But the hatchability difference found between eggs of young and old breeder flocks is still difficult to explain. Meijerhof *et al.* (1994) speculated that the reduced fertility and hatchability with increasing bird age might be related to a decline in the ability of the hens to retain spermatozoa in the sperm storage tubules, a lower quality of the follicle, or pre-ovulatory aging of the follicle. As shown earlier these factors probably influence embryo viability and therefore the ability of the embryo to withstand sub optimal storage conditions. It may be that it is more difficult for the embryo to regulate the pH of its micro environment when albumen and eggshell quality decrease with the increasing age of the breeder flock. Under these conditions, carbon dioxide loss will result in albumen pH increase.

Mayes and Takeballi (1984) reviewed the results of other studies that examined the rate of pre-warming of hatching eggs just prior to setting. Most authors cited by Mayes and Takeballi (1984) pre-warmed eggs for 18 to 24 hours at room temperature (Funk and Forward, 1960; Proudfoot, 1966). The positive effect of this treatment was not consistent. In the study of Meijerhof *et al.* (1994) and in those reviewed by Mayes and Takeballi (1984) the rate of pre-warming was longer than 16 hours. No information in the published literature can be found regarding the effect of shorter pre-warming durations on hatchability and chick quality. It can be hypothesised that when the rate of pre-warming is high, eggs are exposed to sub-optimal incubation temperatures for a short period of time. When the rate of pre-warming is low (12 to 24 hours) at the beginning of the incubation process, mitosis occurs at sub-optimal temperatures for a certain period of time, and abnormal or delayed embryonic development may occur. Another possibility is that the rate of duplication of cells is low and consequently, less viable embryonic cells are available to form an effective barrier between the inside of the embryo and the exterior, or to produce sufficient carbon dioxide, to reduce the pH level to a level of 8.2 in the micro environment of the embryo during the first few days of incubation. The negative effects of a low rate of pre-warming may be exacerbated in

embryos from eggs stored for prolonged periods those stored for short periods. This is because prolonged stored embryos probably contain less viable cells than short stored embryos. To investigate whether these hypothesis are true, more research is needed.

When the embryo is not able to regulate the pH level in its micro environment during early incubation, high carbon dioxide concentrations in the incubator may help to optimise the pH level in the micro environment of the embryo. If the carbon dioxide pressure outside the egg is higher than inside the egg, the loss of carbon dioxide from the eggs halts and the albumen pH may decrease as a result of the carbon dioxide production from the embryo. Whether high carbon dioxide levels in the incubator during early incubation are positive for embryonic development has been reviewed by Onagbesan *et al.* (2007) and is still an important topic for research (De Smit, L. *et al.,* 2006; Bruggeman *et al.,* 2007).

Conclusions

It can be concluded that during storage cell death occurs and/or embryo viability is negatively affected. The cause of this is not known, but the length of storage period and changes in the egg characteristics are two contributing factors. Embryo viability seems to be dependent on the total number of viable cells, the developmental stage of the embryo and the pH level of the micro environment of the embryo. During short storage periods the developmental stage of the embryo may not be important in the maintenance of embryo viability, probably because cell death is low. However, during prolonged storage periods, the developmental stage of the embryo may hold greater importance in maintaining the population of viable cells. An embryo in an advanced, but quiescent, developmental stage may be able to form an effective barrier against the alkaline albumen and might be able to produce sufficient amounts of carbon dioxide to reduce the pH level via its own metabolism. If this is true, the changes in the egg characteristics occurring during storage, such as water loss from the egg, do not affect embryonic development as long as the embryo is able to optimize the pH of its own micro environment. When the embryo is not able to regulate this, altering the atmosphere during storage and/or early incubation seems to be crucial for the optimal start of embryonic development. More research is needed to investigate whether, and under, what circumstances, the embryo is able to regulate the pH of its own micro environment sufficiently. For situations where the embryo is not able to regulate micro environment pH more research is needed to investigate the optimal atmosphere during storage and early incubation.

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References

ARORA, K.L. and KOSIN, I.L. (1966) Changes in the gross morphological appearance of chicken and turkey blastoderms during preincubation storage. *Poultry Science* **45**: 819-825.

ARORA, K.L. and KOSIN, I.L. (1968) The response of the early chicken embryo to pre-incubation temperature as evidenced from its gross morphology and mitotic pattern. *Physiological Zoology* **41**: 104-112.

ASMUNDSON, V.S. and MACILRAITH, J.J. (1948) Preincubation tests with turkey eggs. *Poultry Science* **27**: 394-401.

BAKST, M.R. and AKUFFO, V. (1999) Impact of egg storage on embryonic development. *Avian and Poultry Reviews* **13**: 125-131.

BAKST, M.R. and HOLM, L. (2003) Impact of egg storage on carbonic anhydrase activity during early embryogenesis in the turkey. *Poultry Science* **82**: 1193-1197.

BAKST, M.R. and HOWARTH, B. Jr. (1977) Hydrolysis of the hen's perivitelline layer by cock sperm. *Biology of Reproduction* **17**: 370-379.

BECKER, W.A. (1964) The storage of white leghorn hatching eggs in plastic bags. *Poultry Science* **43**: 1109-1112.

BECKER, W.A. and BEARSE, G.E. (1958) Pre-incubation warming and hatchability of chicken eggs. *Poultry Science* **37**: 944-948.

BECKER, W.A., SPENCER, J.V. and SWARTWOOD, J.L. (1968) Carbon dioxide during storage of chicken and turkey hatching eggs. *Poultry Science* **47**:251-258.

BENTON, C.E. and BRAKE, J. (1996) The effect of broiler breeder flock age and length of egg storage on egg albumen during early incubation. *Poultry Science* **75**: 1069-1075.

BERG. L.R. (1945) The relationship of clutch position and time interval between eggs to eggshell quality. *Poultry Science* **24**: 555-563.

BLOOM, S.E., MUSCARELLA, D.E., LEE, M.Y. and RACHLINSKI, M. (1998) Cell death in the avian blastoderm: resistance to stress-induced apoptosis and expression of anti-apoptotic genes. *Cell Death Differentiation* **5**: 529-538.

BOWLING, J.A. and HOWARTH, B. (1981) The effects of exposing broiler breeder eggs to high temperatures before storage on hatchability and subsequent performance of chicks. *Poultry Science* **60**: 2333-2336.

BRAKE, J., WALSH, T.J. and VICK, S.V. (1993) Hatchability of broiler eggs as influenced by storage and internal quality. *Zootechnica International* **16**: 30-41.

BRILLARD, J.P. (1993) Sperm storage and transport following natural mating and artificial insemination. *Poultry Science* **72**: 923-928.

BRILLARD, J.P. and MCDANIEL, G.R. (1986) Influence of spermatozoa numbers and insemination frequency on fertility in dwarf broiler breeder hens. *Poultry Science* **65**: 2330-2334.

BRUGGEMAN, V., WITTERS, A., DE SMIT, L., DEBONNE, M., EVERAERT, N., KAMERS, B., ONAGBESAN, O.M., DEGRAEVE, P. and DECUYPERE, E. (2007) Acid-base balance in chicken (*Gallus domesticus*) incubated under high CO_2 concentrations during the first 10 days of incubation. *Respiration Physiology and Neurobiology* **159**: 147-154.

BURLEY, R.W. and VADEHRA, D.V. (1989) *The avian egg, chemistry and biology*, pp. 472 (New York, John Wiley and Sons).

COLEMAN, J.W. and SIEGEL, P.B. (1966) Selection of body weight at eight weeks of age. 5. Embryonic state at oviposition and its relationship to hatchability. *Poultry Science* **45**: 1003-1011.

DE SMIT, L., BRUGGEMAN, V., TONA, J.K., DEBONNE, M., ONAGBESAN, O., ARCKENS, L., DE BAERDEMAEKER, J. and DECUYPERE, E. (2006) Embryonic developmental plasticity of the chick: increased CO_2 during early stages of incubation changes the developmental trajectories during prenatal and postnatal growth. *Comparative Biochemistry and Physiology A, Molecular and Intergrative Physiology* **145**: 166-175.

EDWARDS, C.L. (1902) The physiological zero and the index of development for the eggs of the domestic fowl *Gallus domesticus*. *American Journal of Physiology* **6**: 351-396.

EYAL-GILADI, H. (1984) The gradual establishment of cell commitments during the early stages of chick development. *Cell Differentiation* **14**: 245-255.

EYAL-GILADI, H. (1991) The early embryonic development of the chick as an epigenetic process. *Critical Review of Poultry Biology* **3**: 143-166.

EYAL-GILADI, H. and KOCHAV, S. (1976) From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of development of the chick. I. General morphology. *Developmental Biology* **49**: 321-337.

FASENKO, G.M., HARDIN, R.T. and ROBINSON, F.E. (1992a) Relationship of hen age and egg sequence position with fertility, hatchability, viability, and pre-incubation embryonic development in broiler breeders. *Poultry Science* **71**: 1374-1383.

FASENKO, G.M., ROBINSON, F.E. and HARDIN, R.T. (1992b) Research Note: variability in pre-incubation embryonic development in domestic fowl. 2. Effect of duration of egg storage period. *Poultry Science* **71**: 2129-2132.

FASENKO, G.M., WILSON, J.L., ROBINSON, F.E. and HARDIN, R.T. (1999) Effects of length of egg nest holding time and high environmental temperatures on pre-storage embryonic development, survival, and hatchability of broiler breeders. *Journal of Applied Poultry Research* **8**: 488-492.

FASENKO, G.M., CHRISTENSEN, V.L., WINELAND, M.J. and PETITE, J.N. (2001a) Examining the effects of pre-storage incubation of turkey breeder eggs on embryonic development and hatchability of eggs stored for four to fourteen days. *Poultry Science* **80**: 132-138.

FASENKO, G.M., ROBINSON, F.E., WHELAN, A.I., KREMENIUK, K.M. and WALKER, J.A. (2001b) Pre-storage incubation of long-term stored broiler breeder eggs: 1. Effects on hatchability. *Poultry Science* **80**: 1406-1411.

FROMM, D. (1964) Strength distribution, weight and some histological aspects of the vitelline membrane of the hen's egg yolk. *Poultry Science* **43**: 1240-1246.

FROMM, D. (1966) The influence of ambient pH on moisture content and yolk index of the hen's yolk. *Poultry Science* **45**: 374-379.

FUNK, E.M. and BELLIER, H.V. (1944) The minimum temperature for embryonic development in the domestic fowl. *Poultry Science* **23**: 538-540.

FUNK, E.M. and FORWARD, J. (1960) Effect of holding temperature on hatchability of chicken eggs. *Missouri agricultural experiment station bulletin* **732**: 3-12.

GILLESPIE, J.I. and MCHANWELL, S. (1987) Measurement of intra-embryo pH during early stages of development in the chick embryo. *Cell and Tissue Research* **247**: 445-451.

GOODRUM, J.W., BRITTON, W.M. and DAVIS, J.B. (1989) Effect of storage conditions on albumen pH and subsequent hard-cooked egg peelability and albumen shear strength. *Poultry Science* **68**: 1226-1231.

GUPTA, S.K. and BAKST, M.R. (1993) Turkey embryo staging from cleavage through hypoblast formation. *Journal of Morphology* **217**: 313-325.

HAYS, F.A. and NICOLAIDES, C. (1934) Variability in development of fresh-laid hen eggs. *Poultry Science* **13**: 74-90.

HOWARTH, B. Jr, and DIGBY, S.T. (1973) Evidence for the penetration of the vitelline membrane of the hen's ovum by a trypsin-like acrosomal enzyme. *Journal of Reproduction and Fertility* **33**: 123-125.

HURNIK, G.I., REINHART, B.S. and HURNIK, J.F. (1978) Relationship between albumen quality and hatchability in fresh and stored eggs. *Poultry Science* **57**: 854-857.

JANSONIUS, F.A.T., PUTIRULAN, F.F. and KALTOFEN, R.S. (1976) De delingsaktiviteit in het blastoderm van een aantal broedeieren, afkomstig van White Plymouth Rock hennen onmiddelijk na leg bepaald. *Intern rapport pluimveeonderzoek "Het Spelderholt",* Beekbergen.

KAUFMAN, L. (1938) Entwicklung und Wachstum von Hühnerembryonen in frischen und in gelagerten Eiern. *Archiv für Geflügelkunde* **13**: 63-77.

KOSIN, I.L. and ARORA, K.L. (1966) The pattern of early embryonic development in two genetically isolated lines of Broad Breasted Bronze turkeys. *Poultry Science* **45**: 622-629.

LAPAO, C., GAMA, L.T. and CHAVEIRO SOARES, M. (1999) Effects of broiler breeder age and length of egg storage on albumen characteristics and hatchability. *Poultry Science* **78**: 640-645.

LODGE, J.R., FECHHEIMER, N.S. and JAAP, R.G. (1971) The relationship of *in vivo* sperm storage interval to fertility and embryonic survival in the chicken. *Biology of Reproduction* **5**: 252-257.

LOURENS, A. (2006) Heating eggs before storage increases hatchability. *World Poultry* **22**(4): 22-23.

LOURENS, A., VAN DEN BRAND, H., MEIJERHOF, R. and KEMP, B. (2005) Effect of eggshell temperature during incubation on embryonic development, hatchability and posthatch development. *Poultry Science*, **84**: 914-920.

MAYES, F.J. and TAKEBALLI, M.A. (1984) Storage of eggs of the fowl (*Gallus domesticus*) before incubation: a review. *World's Poultry Science Journal* **40**: 131-140.

MATHER, C.M. and LAUGHLIN, K.F. (1976) Storage of hatching eggs: the effect on total incubation period. *British Poultry Science* **17**: 471-479.

MCKERLEY, R.G., NEWELL, G.W., BERRY, J.G., ODELL, G.V. and MORRISON, R.D. (1967) The effects of some acidic and alkaline atmospheres on the changes in pH and haugh units in chicken eggs. *Poultry Science* **46**: 118-132.

MCNALLY, E.H. (1943) Some characteristics of the ovomucin gel of egg white. *Poultry Science* **22**: 25-29.

MEIJERHOF, R. (1994) Theoretical and empirical studies on temperature and moisture loss of hatching eggs during the pre-incubation period. *Ph. D. Thesis*, Landbouwuniversiteit Wageningen.

MEIJERHOF, R., NOORDHUIZEN, J.P.T.M. and LEENSTRA, F.R. (1994) Influence of pre-incubation treatment on hatching results of broiler breeder eggs produced at 37 and 59 weeks of age. *British Poultry Science* **35**: 249-257.

NALBANDOV, A. and CARD, L.E. (1943) Effect of stale sperm on fertility and hatchability of chicken eggs. *Poultry Science* **22**: 218-226.

NECHAEVA, M.V., TOHARDT, H., HUHNKE, A., MAKARENKO, I.G. and TURPAEV, T.M. (2004) Effects of environmental factors on the amnion rhythmic contractions in chick embryogenesis. *Avian and Poultry Biology Reviews* **15**: 137-144.

OLSON, M.W. and FRAPS, R.M. (1944) Maturation, fertilization and early cleavage of the egg of the domestic turkey. *Journal of Morphology* **74**: 297-309.

ONAGBESAN, O., BRUGGEMAN, V., DE SMIT, L., DEBONNE, M., WITTERS, A., TONA, K., EVERAERT, N. and DECUYPERE, E. (2007) Gas exchange during storage and incubation of avian eggs: effects on embryogenesis, hatchability, chick quality and post-hatch growth. *World's Poultry Science Journal* **63**: 557-573.

PROUDFOOT, F.G. (1962) The decline of internal egg quality during storage at 30°F and 70°F among six strains of Leghorns reared in confinement and on range. *Poultry Science* **41**: 98-103.

PROUDFOOT, F.G. (1964) The effects of plastic packaging and other treatments on hatching eggs. *Poultry Science* **43**: 87-95.

PROUDFOOT, F.G. (1965) The effect of film permeability and concentration of nitrogen, oxygen and helium gases on hatching eggs stored in polyethylene and Cryovac bags. *Poultry Science* **44**: 636-644.

PROUDFOOT, F.G. (1966) Hatchability of stored eggs as affected by daily turning during storage and pre-warming and vacuuming eggs enclosed in plastic with nitrogen. *Canadian Journal of Animal Science* **46**: 47-50.

PROUDFOOT, F.G. (1972) Influence of an improved hatching-egg storage method on the subsequent performance of broiler chickens. *Canadian Journal of Animal Science* **52**(2): 303-308.

RADATZ, E., EYAL-GILADI, H. and KUCERA, P. (1987) Patterns of oxygen consumption during establishment of chephalocaudal polarity in the early chick embryo. *Journal of Experimental Zoology* **Supplement 1**: 213-218.

REINHART, B.S. and HURNIK, G.I. (1982) Hatching performance of cryovac enclosed hatching eggs stored in high humidity environment. *Poultry Science* **61**: 564-566.

RENEMA, R.A., FEDDES, J.J.R., SCHMID, K.L., FORD, M.A. and KOLK, A.R. (2006) Internal egg temperature in response to pre-incubation warming in broiler breeder and turkey eggs. *Journal of Applied Poultry Research* **15**: 1-8.

ROBINSON, F.E. (2002) Management for control of ovarian development in broiler breeders. *Avian Medicine* **59**: 1-7.

ROBINSON, F.E., HARDIN, R.T., ROBINSON, N.A. and WILLIAMS, B.J. (1991) The influence of egg sequence position on fertility, embryo viability and embryo weight in broiler breeders. *Poultry Science* **70**: 760-765.

SADLER, W.W. (1955) Chronological relationship of the disappearance of the vitelline membrane and the closure of the amnio-chorion in avian embryos and its implications. *Poultry Science* **34**: 754-760.

SCOTT, T.A. and SILVERSIDES, F.G. (2000) The effect of storage and strain of hen on egg quality. *Poultry Science* **79**: 1725-1729.

SCOTT, H.M. and WARREN, D.C. (1936) Influence of ovulation rate on the tendency of the fowl to produce eggs in clutches. *Poultry Science* **15**: 381-389.

SHENSTONE, F.S. (1968) The gross composition, chemistry and physico-chemical basis of organization of the yolk and white. In: CARTER, T.C. (Ed) *Egg quality: a study of the hen's egg,* pp. 26-58 (Edinburgh, Oliver and Boyd).

SILVERSIDES, F.G. and SCOTT, T.A. (2001) Effect of storage and layer age on quality of eggs from two lines of hens. *Poultry Science* **80**: 1240-1245.

SPRATT, N.T. and HAAS, H. (1960) Integrative mechanisms in development of the early chick blastoderm. I. Regulative potentiality of separated parts. *Journal of Experimental Zoology* **145**: 97-137.

STEINKE, L. (1969) Weitere Keimscheibeuntersuchungen an unbebrüteten Hühnereiern. *Archiv für Geflügelkunde* **33**: 133-146.

STEINKE, L. (1972) Keimscheibeuntersuchungen an Hühnereiern unter besonderer Berücksichtigung des Entwicklungszustandes. *Archiv für Geflügelkunde* **36**: 5-10.

STERN, C.D. (1991) The sub-embryonic fluid of the domestic fowl and its relationship to the early development of the embryo. In: TULLET, S.G. (Ed) *Avian incubation*, pp. 81-90 (London, Butterworth-Heinemann).

TANEJA, G.C. and GOWE, R.S. (1962) Effect of varying doses of undiluted semen on fertility and hatchability in the domestic fowl. *Journal of Reproduction and Fertility* **4**: 161-174.

TONA, K., BAMELIS, F., DE KETELAERE, B., BRUGGEMAN, V., MOREAS, V.M.B., BUYSE, J., ONAGBESAN, O. and DECUYPERE, E. (2003) Effects of egg storage time on spread of hatch, chick quality, and chick juvenile growth. *Poultry Science* **82**: 736-741.

WALSH, T.J., RIZK, R.E. and BRAKE, J. (1995) Effects of temperature and carbon dioxide on albumen characteristics, weight loss and early embryonic mortality of long stored hatching eggs. *Poultry Science* **74**: 1403-1410.

WARREN, D.C. and SCOTT, H.M. (1935a) The time factor in egg formation. *Poultry Science* **14**: 195-207.

WARREN, D.C. and SCOTT, H.M. (1935b) Physiological factors influencing the rate of egg formation in the domestic hen. *Journal of Agricultural Research* **51**: 565-572.

WILLIAMS, K.C. (1992) Some factors affecting albumen quality with particular reference to haugh unit score. *World's Poultry Science Journal* **48**: 5-16.

WILSON, H.R. (1991) Physiological requirements of the developing embryo: temperature and turning. In: TULLET, S.G. (Ed) *Avian Incubation*, pp 145-156 (London, Butterworths).

WIT, A.A.C. DE and KRUIP, Th, A.M. (2001) Bovine cumulus-oocyte-complex-quality is reflected in sensitivity for α-amanitin, oocyte-diameter and developmental capacity. *Animal Reproduction Science* **65**: 51-65.

WHITTOW, G.C. (1986) Regulation of body temperature. In: STURKIE, P.D. (Ed) *Avian Physiology*, 4th edition, pp. 221-268 (New York, Springer-Verlag).