Storage of Hatching Eggs

Effects of storage and early incubation conditions on egg characteristics, embryonic development, hatchability, and chick quality

Inge Reijrink

Thesis committee

Thesis supervisor
Prof. dr. ir. B. Kemp
Professor of Adaptation Physiology
Wageningen University

Thesis co-supervisors
Dr. ir. H. van den Brand
Associate professor, Adaptation Physiology Group
Wageningen University

Dr. ing. R. Meijerhof
Poultry consultant
Poultry Performance Plus, Voorst

Other members
Prof. dr. J. Buyse, Catholic University of Leuven, Belgium
Prof. dr. S. Schulte-Merker, Wageningen University
Dr. G. Fasenko, New Mexico State University, Las Cruces, USA
Dr. G. Baggott, University of London, UK

This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS).

Storage of Hatching Eggs

Effects of storage and early incubation conditions on egg characteristics, embryonic development, hatchability, and chick quality

Inge Reijrink

Thesis

Submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 30 June 2010
at 1.30 p.m. in the Aula.

Inge Reijrink
Storage of hatching eggs – Effects of storage and early incubation conditions on egg characteristics, embryonic development, hatchability, and chick quality

Thesis, Wageningen University, Wageningen, NL (2010)
With references, with summaries in Dutch and English

ISBN: 978-90-8585-665-8

Abstract

Reijrink, I.A.M. (2010). Storage of hatching eggs – Effects of storage and early incubation conditions on egg characteristics, embryonic development, hatchability, and chick quality. PhD thesis Wageningen University, the Netherlands.

It is well known that an increase in the storage duration increases incubation duration and decreases hatchability and chick quality. The negative effects of prolonged egg storage (> 7 days) may be caused by changes in the embryo, in the egg characteristics, or by both. The first aim of the current thesis was to investigate which physiological mechanisms are involved in the negative effects of prolonged egg storage on hatchability and chick quality. The second aim was to investigate how these negative effects of prolonged egg storage can be reduced by making changes in storage or early incubation conditions. Treatments, such as prestorage incubation, frequent warming during storage, a change in the storage air composition, different preincubation warming profiles, and hypercapnic incubation during the first 5 days of incubation were used in the current thesis to gain more insight in the cause of the negative effects of prolonged egg storage. Prestorage incubation and frequent warming during storage increased the stage of embryonic development and the number of viable embryonic cells. The effect of these treatments on hatchability was nihil, positive or negative and seems to depend on the stage of embryonic development before and after the treatment. The storage air compositions, studied in the current thesis did not affect embryonic development, hatchability, or chick quality, when eggs were stored for 14 days. This suggests that changes in albumen quality during storage do not affect hatchability and chick quality. The 24-h preincubation warming profile decreased embryonic mortality during the first 9 days of incubation in comparison with the 4-h preincubation warming profile when eggs were stored for 13 days. Hypercapnic incubation during the first 5 days of incubation decreased albumen pH during early incubation, but did not improve hatchability. In conclusion, embryo characteristics seem to have a more important role in the negative effects of prolonged egg storage than changes in the egg characteristics, such as changes in the albumen pH and albumen height.

Key words: egg storage, embryonic development, albumen quality, hatchability, chick quality

Voorwoord

Na vier en half jaar hard werken is het eindelijk zover. Mijn proefschrift is klaar. Onderzoek heeft altijd een open einde, nu sluit ik mijn deel eraan toch een beetje af. Het begin van dit proefschrift is gemaakt in 2004 tijdens het uitvoeren van mijn groot afstudeervak bij de leerstoelgroep Adaptatiefysiologie. Ondanks dat ik kippen enge beesten vond, wist Henry van den Brand me enthousiast te maken voor een afstudeervak waarin het broedproces centraal stond. Na één week literatuur lezen, realiseerde ik me hoe wonderlijk het is dat er na 21 dagen broeden een kuiken uit een ei tevoorschijn komt. Ik wil in de eerste plaats Henry dan ook hartelijk bedanken voor het feit dat hij me enthousiast heeft gemaakt voor het broedproces. Daarmee is tenslotte alles begonnen.

Na het afronden van mijn studie Zoötechniek was een baan op de onderzoeksafdeling van HatchTech Incubation Technology in Veenendaal de volgende stap. Hier kreeg ik na een half jaar de mogelijkheid om aan dit promotieonderzoek te beginnen. Ik ben aan deze uitdaging begonnen zonder echt te weten wat het inhield. Voor Tjitze Meter, directeur van HatchTech, gold echter hetzelfde. Ik ben hem dan ook dankbaar voor het feit dat ik aan dit promotieonderzoek mocht beginnen. Daarnaast wil ik hem bedanken voor het vertrouwen, voor zijn ideeën, voor zijn hulp en voor de financiering.

Zonder een goed woordje van mijn begeleiders Henry van den Brand, Bas Kemp en Ron Meijerhof had ik de baan bij HatchTech waarschijnlijk nooit gekregen, dus ook daarvoor bedankt! Gedurende het project heb ik ontzettend veel gehad aan jullie begeleiding. Bas, je hield de essentie van het onderzoek nauwgezet in de gaten. Ron, je hebt me veel geleerd over de natuurkundige kant van het broeden en je passie voor het schrijven heeft mij enorm gestimuleerd. Henry, je enthousiasme werkt aanstekelijk en je kritische blik heeft een positieve bijdrage geleverd aan dit proefschrift! Jullie inzet en hulp tijdens de experimenten, het delen van jullie kennis en ervaringen, jullie luisterend oor, het duwtje in mijn rug als ik te lang ergens in bleef steken, maar ook zeker het intrappen van de rem als ik teveel van mezelf eiste, waren erg waardevol.

Dan is er nog iemand die vanaf het begin nauw betrokken is geweest bij mijn project en dat is Roos Molenaar. Roos, het was fijn dat we de uitdaging van een promotieonderzoek samen konden aangaan. We hebben heel wat uren samen doorgebracht. Je hield me altijd scherp en ik heb veel van je geleerd. Tijdens experimenten stond je op de gekste tijden voor me klaar. Je bent een kleurrijk persoon die het overal gezellig maakt. Roos bedankt voor alles!

Alle collega's van HatchTech en Adaptatiefysiologie wil ik ook niet ongenoemd laten. Iedereen heeft op zijn manier een steentje bijgedragen aan mijn proefschrift. Bedankt voor jullie hulp bij het voorbereiden en uitvoeren van mijn experimenten en voor de gezelligheid tijdens de koffiepauzes en personeelsuitjes. Een aantal mensen wil ik apart noemen. Cor Termaat en

Stefan Achterberg geweldig dat de technische snufjes altijd net op tijd klaar waren. Ilona van den Anker met jou en de radio erbij was het altijd gezellig en kwam alles in orde! Herken je harige Harrie 10 en 23 op de voorkant van dit proefschrift? Marcel Heetkamp je support bij het gebruik van de klimaat respiratie cellen was onmisbaar. Albert Goorts, Conny Maatjens en Willem van den Oetelaar zonder jullie hulp bij de experimenten was het allemaal niet gelukt.

Ook waren er een aantal studenten betrokken bij de uitvoering van de experimenten. Florian van der Hoeven, Debbie Berghmans, Annelies van der Linden, Linda Baarslag, Gilian van Duijvendijk, Sonja de Vries, Ewoud Hazejager, en Woutrien de Bruin zonder jullie hulp en flexibiliteit had ik het niet gered!

Het eerste jaar van mijn promotieonderzoek heb ik rondgelopen op de afdeling Anatomie en Embryologie van de Universiteit van Maastricht. Ik wil iedereen die daar in die periode werkzaam was bedanken voor de hulp, gastvrijheid en gezelligheid en Henny van Straaten in het bijzonder voor het delen van zijn kennis en voor zijn hulp bij de ontwikkeling van een aantal methoden.

Tevens wil ik Anja Taverne-Thiele van de leerstoelgroep Cel Biologie en Immunologie bedanken voor haar hulp bij de FACS analyses tijdens mijn laatste experiment. Daarnaast wil ik ook Jaap Keijer van de leerstoelgroep Fysiologie van Mens en Dier bedanken voor zijn bijdrage aan de algemene discussie van dit proefschrift.

Bij alle experimenten die ik uitvoerde, was broederij Lagerwey betrokken. Ik wil dan ook alle medewerkers van broederij Lagerwey bedanken voor hun gastvrijheid, voor hun flexibiliteit en voor hun hulp bij mijn experimenten. Zonder jullie had ik de experimenten nooit kunnen uitvoeren! Tevens wil ik alle medewerkers van Demetris Diergezondheid bedanken voor de prettige samenwerking.

Men zegt vaak dat de laatste loodjes het zwaarst wegen. Ook bij mij bleek dit zo te zijn. Voor het laatste experiment had ik netjes een planning gemaakt, maar op de eerste dag van het experiment bleek al dat ik de hoeveelheid werk zwaar had onderschat. Als dat gebeurt, is het super fijn dat niet alleen je collega's, maar ook je vrienden en familie behulpzaam en flexibel zijn. Dus bij deze wil ik mijn vrienden en familie bedanken voor hun hulp tijdens deze proef, maar natuurlijk ook voor de afleiding buiten het werk! Sorry dat ik op een aantal bruiloften een beetje afgeleid was en dat ik zelfs een vrijgezellenfeest heb moeten missen!

Pap, bedankt dat je altijd achter me staat en me onvoorwaardelijk helpt mijn doelen te bereiken.

Marieke, bedankt voor het maken van de mooie tekeningen die eigenlijk op de voorkant van dit proefschrift hadden moeten komen. Nogmaals sorry dat ik ze niet heb gebruikt!

Dan blijft mijn beste maatje nog over. Paul, ik kan me de eerste keer dat je me hielp bij een experiment nog goed herinneren. Het was op Hemelvaartsdag van het jaar 2007, eigenlijk voor ons allebei een vrije dag. Ik had je gestrikt met de woorden: "Het duurt maar een paar uur en als

we klaar zijn trakteer ik je op een Big Mac." Om 7 uur 's ochtends stonden we vol goede moed samen eieren open te breken en om 7 uur 's avonds zaten we pas bij de McDonalds. Hoezo een paar uur? Na dit voorval heb ik je een hele tijd niet meer om hulp durven vragen, maar bij mijn laatste proef heb je me noodgedwongen toch weer een aantal keren uit de brand geholpen. Paul, bedankt dat je altijd voor me klaar staat en dat je me gestimuleerd hebt om het promotie onderzoek af te maken!

Dan wil ik graag als laatste mijn proefschrift opdragen aan "ons mam". Mam, in het eerste jaar van mijn studie aan Wageningen Universteit heb ik helaas afscheid van je moeten nemen door een ongeneeslijke ziekte. Je hebt vroeger door omstandigheden nooit de kans gehad om te studeren, terwijl je dat eigenlijk heel graag had gewild. Dit was voor mij mede een drijfveer om aan dit promotieonderzoek te beginnen en om het goed af te ronden. Mam, bedankt voor de leergierigheid die ik van je heb meegekregen! Dan is het vanaf vandaag geen Trees meer maar Dr. Trees.

Contents

General Introduction

After oviposition, hatching eggs are first stored at the breeder farm, then transported to the hatchery and stored again before they are finally set in the incubator. The storage duration depends on the supply of hatching eggs, hatchery capacity, and market demand for day-old chicks. Normally, commercial hatcheries set their eggs after 3 to 5 days of storage to minimize the negative effects of egg storage on hatchability and chick quality. However, a hatchery may need to increase the storage duration in some situations. Especially in hatcheries that incubate eggs of grand parent stocks, storage durations beyond 7 days occur often (Proudfoot, 1969). It is well known that an increase in the storage duration increases incubation duration (Mather and Laughlin, 1976; Tona et al., 2003), decreases hatchability (Becker, 1964; Merritt, 1964; Fasenko et al., 2001b; Tona et al., 2004; Yassin et al., 2008), decreases chick quality on the day of hatch (Byng and Nash, 1962; Tona et al., 2003, 2004), decreases subsequent growth performance (Becker, 1960; Merritt, 1964; Tona et al., 2003, 2004), and increases posthatch mortality (Merritt, 1964; Yassin et al., 2009). A storage duration beyond 7 days has a pronounced effect on hatchability (Yassin et al., 2008). On average, each extra day of storage up to the seventh day of storage reduces hatchability by 0.2%, whereas this percentage increases to 0.5% after the seventh day of storage (Yassin et al., 2008). A simple calculation shows that the negative effect of prolonged egg storage on hatchability has a substantial effect on the economic returns of a hatchery in the Netherlands on a yearly basis. In 2007, about 607,000,000 broiler eggs were set in the Netherlands (PVE, 2008) and their average hatchability of set eggs was 81%. In 2007, the average price of a day-old chick was € 0.275 (Horne, 2009). It can be assumed that 5% of all eggs were stored for 10 days instead of 5 days, which reduces their hatchability by 1.9% (2 x 0.2% plus 3 x 0.5%; Yassin et al., 2008). In this situation, 576,650 embryos die during the incubation process due to prolonged egg storage, which represents a loss of € 158,579. In fact, the economic losses are probably higher because the higher posthatch mortality (Merritt, 1964; Yassin et al., 2009) and the lower subsequent growth performance (Becker, 1960; Merritt, 1964; Tona et al., 2003, 2004) caused by prolonged egg storage are not taken into account in this calculation.

Although the negative effects of prolonged egg storage are well known, it is not totally understood why this extended storage has negative effects on hatchability and chick quality (Meijerhof, 1992; Tona et al., 2004). During storage, eggs are stored below temperatures at which morphological development occurs (Funk and Biellier, 1944). However, some cellular activity still occurs in the embryo, such as mitosis and cell death (Arora and Kosin, 1968). In addition to the changes in the embryo, modifications in the egg characteristics (micro- environment of the embryo) also occur during storage. After oviposition, CO_2 and H_2O are lost from the egg, the

albumen pH increases from about 7.6 to about 9.0 (Lapão et al., 1999), yolk pH increases from about 6.0 to about 6.5 (Shenstone, 1968), albumen height decreases (Silversides and Scott, 2001), and strength of the yolk membrane decreases as well (Fromm, 1966). Because the described changes both in the embryo and in the egg characteristics (micro-environment of the embryo) occur during egg storage, it is difficult to distinguish which of these changes are involved in the negative effects of prolonged egg storage on hatchability and chick quality. To investigate whether the changes in the embryo are involved in the negative effects of prolonged egg storage, several authors increased the stage of embryonic development by using prestorage incubation (Kosin, 1956; Becker and Bearse, 1958; Bowling and Howarth, 1981; Proudfoot and Hulan, 1982; Meir and Ar, 1998; Fasenko et al., 2001a, b). To investigate whether the changes in the egg characteristics (micro-environment of the embryo) are involved in the negative effects of prolonged egg storage, several authors have changed the storage air composition by increasing the CO_2 in the storage room (Kosin and Konishi, 1973) or by storing eggs in plastic bags with or without additional CO_2 or N_2 (Becker et al., 1963; Proudfoot, 1964a, b; Krueger et al., 1965; Warren et al., 1965; Gordon and Siegel, 1966; Becker et al., 1967, 1968). In most of the above mentioned studies, only the effect on hatchability was determined, without measuring the changes in the embryo and in the egg characteristics. However, these two latter measurements may clarify why prolonged egg storage negatively affects hatchability and chick quality. Some authors measured the changes in albumen height and albumen pH (albumen quality) during storage or early incubation to understand some of the negative effects of prolonged egg storage (Benton and Brake, 1996; Lapão et al., 1999), but these authors did not alter albumen quality to investigate the effect on embryonic development and hatchability. Therefore, it is unclear whether albumen quality is involved in the negative effects of prolonged egg storage. Furthermore, results of the studies that investigated the effects of storage air composition or prestorage incubation on hatchability were often in conflict with each other. These conflicting results can perhaps be explained by unmeasured differences in embryo viability caused by strain (Yoo and Wientjes, 1991), breeder flock age (Mather and Laughlin, 1979), storage duration (Bloom et al., 1998; Bakst and Akuffo, 1999), or storage conditions (Arora and Kosin, 1968).

The first aim of the current thesis is to investigate which physiological mechanisms are involved in the negative effects of prolonged egg storage on hatchability and chick quality. The second aim is to investigate how these negative effects of prolonged egg storage on hatchability and chick quality can be reduced by making changes in storage or early incubation conditions. Table 1 shows which treatments were used to reduce the negative effects of prolonged egg storage on hatchability and chick quality, and whether the treatments were used to affect the embryo, the egg characteristics (micro-environment of the embryo), or both.

Table 1. Treatments that were used in each chapter to reduce the negative effects of prolonged egg storage on hatchability and chick quality, and whether the treatments were used to affect the embryo, the egg characteristics, or both

Chapters	Embryo	Egg Characteristics
2. Prestorage Incubation	X	
3. Storage Air Composition	X	X
4. Preincubation Warming Profile	X	
5. Prestorage Incubation, Frequent Warming during Storage, and Hypercapnic Incubation	X	X

The results of the different experiments are presented in a chronological order. In Chapter 1, the literature that describes the changes in the embryo and in the egg characteristics (micro-environment of the embryo) during storage and early incubation are reviewed. In addition, it is discussed which changes can be related to the negative effects of prolonged egg storage. Chapter 2 describes two experiments in which prestorage incubation was used to change the stage of embryonic development. The effect of prestorage incubation was investigated on hatchability and chick quality. In the experiment described in Chapter 3, eggs were stored in a CO_2 concentration of 0.74% or 1.50% or an O_2 concentration of 3.0% to affect the conditions in the micro-environment of the embryo. The effects of these treatments on albumen quality, embryonic development, hatchability, and chick quality were measured when the storage duration was 14 days. Chapter 4 describes two experiments in which a 4-h and 24-h preincubation warming profile were compared to determine whether the onset of incubation plays a crucial role in successful embryonic development after short or prolonged egg storage. The effect of preincubation warming profile was examined on embryonic development, hatchability, and chick quality in eggs stored for 4 and 13 or 14 days. In the experiment described in Chapter 5, eggs were exposed to prestorage incubation and frequent warming during storage to increase the stage of embryonic development and/or the number of viable embryonic cells. In addition, eggs were exposed to hypercapnic incubation during the first 5 days of incubation to alter the albumen pH during early incubation. These three treatments were used to investigate the effect on egg characteristics, embryonic development, hatchability, and chick quality. It was also investigated whether the effect of hypercapnic incubation depends on the stage of embryonic development at the onset of incubation. In the general discussion (Chapter 6), the results of the previous chapters are integrated and the factors that possibly cause the negative effects of prolonged egg storage on hatchability and chick quality are discussed.

Chapter 1

The Chicken Embryo and its Micro-Environment during Egg Storage and Early Incubation

I.A.M. Reijrink[1], R. Meijerhof[1], B. Kemp[2], and H. van den Brand[2]

[1]HatchTech Incubation Technology B.V., P.O. Box 256, 3900 AG Veenendaal, the Netherlands;
[2]Adaptation Physiology Group, Wageningen Institute of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands.

Reprinted by the permission of the World's Poultry Science Association
World's Poultry Science Journal (2008) 64:581-598

Summary

When egg storage is beyond 7 days, hatchability and chick quality decline. The reason for this decline has been investigated, but is not completely understood. At oviposition, the developmental stage of the chicken embryo varies and so do the total number of viable embryonic cells. During egg storage, changes occur in the embryo. Embryo viability at the end of storage seems to depend on the number of viable embryonic cells and the stage of embryonic development at oviposition. When the hypoblast is completely formed (a quiescent stage of embryonic development), the embryo seems to be better able to endure prolonged egg storage than embryos that are less or further advanced.

During storage, changes also occur in the egg characteristics, such as albumen viscosity (albumen height), 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 in the range of 7.9 to 8.4 seems to be optimal for embryonic development. Albumen pH may affect embryo viability, but embryo viability may in turn, affect albumen pH. It has been hypothesized that an embryo in which the hypoblast is completely formed is better able to provide an effective barrier between the internal embryo and its exterior (yolk and albumen) and/or is better able to produce a sufficient amount of CO_2, which will reduce the pH in the micro-environment of the embryo to the optimal pH range of 7.9 to 8.4. It appears that, to maintain hatchability and chick quality after prolonged egg storage, the stage of embryonic development should be advanced to the developmental 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 range of 7.9 to 8.4.

Keywords: embryo viability, albumen pH, egg storage, preincubation warming duration, early incubation

Introduction

After eggs are laid at the breeder farm, 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, prewarmed 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) hypothesized that this is caused

by high albumen viscosity (albumen height) in fresh eggs, which impedes oxygen transport to the embryo. A storage duration beyond 7 days at standard storage conditions (10-20°C and 50-80% RH) cause a delay in hatch time (Mather and Laughlin, 1976; Tona et al., 2003) and a decline in hatchability (Becker, 1964; Merritt, 1964; Fasenko et al., 2001b; Tona et al., 2004) and chick quality (Byng and Nash, 1962; Merritt, 1964; Tona et al., 2003, 2004). When eggs are set after prolonged egg storage (> 7 days) embryo viability is reduced. To understand the negative effects of prolonged egg storage on embryo viability, two important questions need to be answered:

1) Which factors affect embryo viability during storage and early incubation?
2) How can the reduction in embryo viability be prevented?

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

Prestorage 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 to 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 to 11 hours in the shell gland, the cytoplasmic mass of the germinal disc cleaves rapidly (Eyal-Giladi, 1991; stages EG1 to EG6 according to Eyal-Giladi and Kochav, 1976 (**EG**)). 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} hours in the shell gland, two distinct regions are formed: the area pellucida, from which the embryo will develop, and the surrounding area opaca, which 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 EG7 to

EG10). The area pellucida consists of 1 to 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. Within hens embryonic development varies 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 EG10. More recent research of Fasenko et al. (1992a, b) confirmed this and reported that when the stage of embryonic development at oviposition was not stage EG10, 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 stages of embryonic development was not standardized at that time. According to Kosin and Arora (1966), the term 'advanced gastrula stage' is the stage of embryonic development in which the embryo has a distinct area opaca and area pellucida (≥ stage EG10). Embryos that 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 stage of embryonic development 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 incubation. Coleman and Siegel (1966) compared the stages of embryonic development at oviposition of a high and a low weight genetic line. They found that the embryos of the high weight line were less-advanced and more sensitive to prolonged egg storage than the embryos of the low weight line. Hays and Nicolaides (1934) and Steinke (1972) assigned hens to three groups based on their hatchability and investigated the stage of embryonic development 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 and very good hatchability contained embryos at an advanced gastrula stage (Table 1). It seems that the stage of embryonic development and hatchability are positively associated, but other factors, such as genetic selection, or differences in optimal incubation conditions may, affect hatchability as well.

Table 1. The variation in the stage of embryonic development of the chicken embryo at oviposition related to hatchability (Adapted from Steinke, 1972).

Pre-gastrula stage (< stage EG10*)	Advanced gastrula stage (≥ stage EG10*)	Hatchability
(% of embryos in this stages of embryonic development)	(% of embryos in this stages of embryonic development)	(%)
30	70	>84
30	70	55-84
62	38	<55

*Stage of embryonic development according to the classification table of Eyal-Giladi and Kochav (1976)

Factors that affect 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 affect 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 of egg laying; called '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 to 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 to 10 hours 'open period' per day, and depends on progesterone production from the mature follicle. The 'open period' is the only time period in which 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 a 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 follicles (Scott and Warren, 1936). It is shown that

embryos in first-of-sequence eggs are further developed at oviposition than embryos in the subsequent eggs (average developmental stage: EG10.36 and EG10.05, respectively; Fasenko et al., 1992a). The reason why the embryo in the first-of-sequence egg is more developed than in subsequent eggs is unknown. The first egg of a sequence may remain longer in the shell gland than subsequent eggs (Berg, 1945) or aging of the follicle on the ovary results in a faster onset of development due to intra-follicular processes. The latter has been seen in bovine cumulus-oocyte-complexes (De Wit and Kruip, 2001). Based on the results of Steinke (1972), it can be concluded that embryonic development at oviposition has a positive relationship with hatchability. Embryos in first-of-sequence eggs are more advanced at oviposition than embryos in subsequent eggs, therefore, it can be assumed that also hatchability of these eggs is higher than 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 may be due to pre-ovulatory aging of the oocyte or changes in yolk composition that affect embryonic growth. Based on this research, it can be concluded that more advanced embryos at oviposition are not always a guarantee for good hatchability. The decline in hatchability observed in older breeder flocks, may 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 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 acrocomal 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 likelihood of fertilization increases when the number of spermatozoa in the perivitelline layer of the yolk increases. Several factors affect 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% when the age of the sperm was between 13 to 16 days and 74.1% when the age of the sperm was between 1 to 12 days. 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 were not affected. Fasenko et al. (1992a) showed that the stage of embryonic development decreased from EG10.3 to EG10 when the days after insemination increased from 2 to 8 days.

Based on these results, it seems that factors that negatively affect fertility may negatively affect the stage of embryonic development 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 may affect embryo viability at oviposition.

The Egg Passage Rate through the Oviduct

Warren and Scott (1935a) measured the total oviduct length of individual hens in 19 hens and found that it ranged between 555 mm to 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 seperate 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 by sequence position, is significant enough to make a difference in embryo viability.

In conclusion, embryo viability and the likelihood that a viable chick will hatch from an egg seems to be associated with the stage of embryonic development at oviposition. The stage of embryonic development at oviposition may be affected 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 affected 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 stage of embryonic development is affected 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 in eggs that remained longer (3.5 to 6.5 hours) in the nests at an environmental temperature of 28°C were more developed than in eggs collected just after oviposition and stored at 18.9°C to 20.7°C for 10 hours (stage EG11.67 and EG10.38, respectively). These results were likely caused by the longer exposure time to temperatures that enhanced embryonic development. Hatchability of fertile eggs and the incidence of early embryonic mortality were not significantly affected by the length of nest holding. In this study, the storage duration 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 effect 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 nest temperature of 30°C was compared to 20°C in a 59 week old breeder flock. They suggested that eggs produced by older birds were more sensitive to high temperatures in the nest boxes than young flocks. These results showed that the viability of the embryo can be affected in the time between oviposition and egg collection. The effect of nest holding temperatures seems to be interrelated with different factors, such as storage duration 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; Merritt, 1964; Fasenko et al., 2001b; Tona et al., 2004) and chick quality (Tona et al., 2003, 2004). An important question that remains unanswered is: what happens to the embryo during storage? As stated earlier, one factor that highly affects embryonic development is temperature. Early research suggested that no developmental changes occured in embryos when eggs were stored at temperatures well below normal incubation temperatures. This temperature was termed 'the physiological zero' and was reported to be either 20°C to 21°C (Edwards, 1902) or 24°C to 27°C (Funk and Biellier, 1944).

Arora and Kosin (1968) did not observe any change in the gross morphology of the embryos during storage up to 21 days when eggs were stored at 7.2°C, 12.8°C, or 18.3°C, but observed changes in the cellular activity of the embryos. When storage duration increased, the number of

mitotic and necrotic indexes increased at all three temperatures, particularly in the 12.8°C and 18.3°C temperature treatments. 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, they 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 embryo was marginal and, therefore, they suggested that a storage temperature of 7.2°C was more suitable for the preservation of viable embryonic cells than 12.8°C or 18.3°C, when storage is prolonged.

Bakst and Akuffo (1999) investigated the total number of embryonic cells of turkey embryos at oviposition and after 2, 4, and 14 days of storage at 18°C. The total number of embryonic cells was 32,000, 21,500, 19,000, and 21,000, respectively. Thus, within the first 48 hours of storage the total number of embryonic cells decreased by 30%. The decrease in the total number of cells 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 and this increased to 13.9% after 14 days of storage at 12°C.

The ability of an embryo to survive storage may vary among domestic avian species, due to differences in embryonic development at oviposition. At oviposition, the stage of embryonic development of the turkey embryo is characterized by the initial appearance of the area pellucida (Gupta and Bakst, 1993), whereas in the chicken embryo the area pellucida is already completely formed (Eyal-Giladi and Kochav, 1976). It is possible that at oviposition, the stage of embryonic development of the turkey embryo is more sensitive than the chicken embryo. Arora and Kosin (1966) showed that some turkey embryos contained numerous vacuoles in the area opaca and pellucida after 1 to 2 days of storage, whereas in chicken embryos this occurred after 14 days of storage. Previous research quantified the number of embryonic cells in chicken (Spratt and Haas, 1960; Raddatz et al., 1987) and turkey embryos (Bakst and Akuffo, 1999) at oviposition. Turkey embryos have much lower number of embryonic cells at oviposition and, therefore, cell death could have a greater impact in this species. Because the total number of embryonic 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 embryonic cells increases, the overall percentage of cell death decreases and embryo viability is maintained.

Prestorage Incubation

Several authors have investigated the effect of prestorage 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. (2001b) 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 afterward. Embryonic development advanced significantly with increasing length of the prestorage incubation period. Hatchability of eggs stored for 4 days was not affected by prestorage incubation. Hatchability of eggs stored for 14 days was significantly better after 6 hours of prestorage incubation than without prestorage incubation. Lourens (2006) subsequently confirmed a positive effect of prestorage incubation on hatchability of broiler breeder eggs. In comparison to the control treatment, the increase in hatchability after 3, 6, and 9 hours of prestorage incubation and a storage duration of 14 days was 9.2% ($P \leq 0.05$), 11.8% ($P \leq 0.05$), and 6.4% ($P > 0.05$), respectively. Hatchability among the three treatments (3, 6, and 9 hours) was not significantly different.

Fasenko et al. (2001b) suggested that there is an optimal stage of embryonic development to endure prolonged egg storage. Improvements in hatchability after prestorage incubation were not simply due to lengthening the total incubation period. The prestorage incubation treatments that improved hatchability of long-term stored eggs, advanced embryos to the stage of embryonic development in which hypoblast formation was complete (stage EG13). These embryos were probably more resistant to prolonged egg storage than embryos that did not complete hypoblast formation (< stage EG13) or embryos that began to form the primitive streak (> stage EG13). The formation of the primitive streak is a period of active cellular migration and differentiation of embryonic cells (Bellairs, 1986). Fasenko et al. (2001b) hypothesized that it was not favourable to store embryos in this developmental stage for a prolonged time because storage would impede critical cellular and embryonic processes. A storage duration of 4 days, however, was not detrimental for embryos that 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) depended upon the length of storage to which they have been exposed. The beneficial effect of prestorage incubation depends, therefore, on the interaction between the stage of embryonic development at oviposition, storage duration, and the length of the prestorage incubation period. The results of Fasenko et al. (2001b) also showed that hatchability after 6 hours of prestorage incubation and 14 days of storage was not comparable to hatchability after 0 hours of prestorage incubation and 4 days of storage. It can be hypothesized, that it is better to maintain the number of viable embryonic cells during storage than to compensate for cell death by using prestorage incubation, which increases the stage of embryonic development and the number of viable embryonic cells.

Egg Characteristics during Storage

During storage, changes occur in the egg characteristics. Because these egg characteristics form the micro-environment surrounding the embryo, it is possible that the changes in the egg characteristics also affect cell death, embryo viability, or both. The changes in the egg characteristics, therefore, merit discussion.

Changes in Albumen pH and Influencing Factors

At oviposition, albumen pH is around 7.6 (Stern, 1991). After oviposition, CO_2 is released from the egg. Due to the release of CO_2, the equilibrium of the carbonate-bicarbonate buffer system is thought to be shifted towards production of CO_2. Consequently, albumen pH rises to a pH around 9.0 after 4 days of storage and does not increase much more thereafter (Lapão et al., 1999). The rise to a pH around 9.0 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 duration, 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 (albumen height) is maximal (Silversides and Scott, 2001) and decreases afterward. 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 CO_2 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 duration, 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 may be necessary so that enough ovomucin remains for the developing embryo during incubation (Hurnik et al., 1978). Albumen and chalazae are protein gels, which consist of ovomucin fibres to which water is bound (Fromm, 1966). McNally (1943) showed that the condition of the ovomucin changed when albumen pH changed. When the pH was in between 6.0 to 6.4 or in between 8.3 to 8.5, the ovomucin existed in the gel form, and at higher pH values, it existed as a viscous solution. These protein gels

seem to have an optimum pH at which the fibres will bind the largest amount of water (Fromm, 1966). Based on the results of McNally (1943) this optimum pH level is about 8.3 to 8.5, which was also found 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 in different atmospheres that affected albumen viscosity and albumen pH.

As previously discussed, albumen pH is around 9.0 after 4 days of storage at standard storage conditions. It is impossible to maintain an albumen pH between 8.3 to 8.5 during prolonged egg storage, without manipulation of the storage conditions. Because a pH of 8.3 to 8.5 seems to be important to maintain albumen viscosity, two question arise:

1) Whether this albumen pH and viscosity are also optimal for maintainance of embryo viability during storage and for embryonic development during early incubation?
2) How the two pH levels of 9.0 (to prevent microbial contamination) and 8.3 to 8.5 (optimal level for albumen viscosity and perhaps embryonic 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 water 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 outside the egg and the yolk. Due to water movements, the osmolarity of the albumen and yolk changes. The loss of water to the environment outside the egg is affected 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 affect on the viscosity of the albumen, although later Benton and Brake (1996) were unable to find a direct relationship between water loss and albumen pH and height.

Meijerhof et al. (1994) showed that water loss between egg collection and day 17 of incubation was not affected by the relative humidity of 55% or 75% during a storage duration of 7 days. Based on these results, they suggested that the effect of water loss during storage on hatching results is limited, under practical conditions. Although the loss of water during storage is minimal compared to 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 around 6.0 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 may 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 at 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 that affects the strength of the vitelline membrane and the yolk index. This is in agreement with earlier findings of Fromm (1964), who hypothesized that the strength of the vitelline membrane highly depend on the quality of the chalaziferous layer surrounding the yolk. The chalaziferous layer is a layer of fibers and a gel-like substance. As stated earlier, the viscosity of a gel highly depends on pH. Because the viteline membrane and chalaziferous layer form the boundary between the embryo and the albumen, the quality of the chalaziferous layer and vitelline membrane are important because they protect the embryo during storage and 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 is on one side in direct contact with the yolk, while on the other side, the embryo touches the IPVL. Consequently, the embryo is close to the chaliziferous layer of the albumen. Therefore, the yolk and chaliziferous layer form the micro-environment of the embryo. After 4 days of storage, when the pH of the chalaziferous layer is around 9.0 and the pH of the yolk is around 6.5 to 6.8, the pH difference across the embryo is 3 pH levels. Benton and Brake (1996) hypothesized 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 a pH of 9.0 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 the optimal micro-environment is still unknown.

Embryo and Egg Characteristics during Storage

Albumen pH increases to a pH around 9.0 within 4 days of storage (Lapão et al., 1999), perhaps to ensure protection against microbial contamination. As stated earlier, a pH around 9.0 does not correspond to the optimal pH for maintenance of the strength of the vitelline membrane, the yolk index, and albumen viscosity (8.3-8.5; McNally, 1943; Fromm, 1966; McKerley et al., 1967). Although the latter was investigated in table eggs, it agrees with the optimal pH in the extracellular space of the embryo after 24 hours of incubation, as found by Gillespie and McHanwell (1987). They investigated the pH in the extracellular space in isolated chicken embryos in vitro at stages 4-22 somites (normally between 26 hours 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 its exterior, and protected the embryo from suboptimal pH levels. It is unknown 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 effective barrier may cause a depletion of energy reserves available to the embryo and may result in embryonic mortality. Raddatz et al. (1987) showed that oxygen fluxes increased in the posterior region of embryos at developmental stage EG10, which is probably connected to the onset of the expansion of the primary hypoblast. As the number of viable embryonic cells increase in this region, the cell density increases as does the metabolic activity. It can be assumed that the CO_2 production of the embryonic cells increased at the same time. A more advanced embryo, which has a higher number of viable embryonic cells, may be better able to form an effective barrier between the inside of the embryo and its exterior and/or may be better able to produce a sufficient amount of CO_2 than a less-advanced embryo. A higher CO_2 production may reduce the pH in the embryo's own micro-environment from a pH around 9.0 to a pH around 8.0. In addition, this may optimize the yolk index, strength of the vitelline membrane, and albumen viscosity. The albumen viscosity that is a result of an albumen pH between 8.3 to 8.5 may also fit to 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 will be maintained around 9.0.

If this hypothesis is correct, the stage of embryonic development, the number of viable embryonic cells, and the pH in 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 EG10) is unable to form an effective barrier between the inside of the embryo and its exterior, nor produce sufficient amounts of CO_2 to regulate the pH of its own micro-environment. In such situation, it is important to alter the storage and early incubation atmosphere to maintain the albumen pH around 8.2.

Manipulation of Egg Environment during Storage

Many authors investigated the effect of altering the storage atmosphere on hatchability. The results of the different studies are not consistent and often difficult to explain. Proudfoot (1964a) investigated the effect of packing eggs in plastic bags (Cryovac and Poly. No. 100) supplemented with an unknown amount of CO_2. The use of supplementary CO_2 in the plastic bags had a severe depressing effect on hatchability. Hatchability of eggs that were packed in the Cryovac bags and supplemented with CO_2 even reduced to zero when the storage duration was 14 and 21 days. Packing eggs in a plastic bag without supplementary CO_2 was beneficial compared to no plastic bags. In two other studies, Proudfoot (1965, 1972) showed via different tests that packing eggs in plastic bags supplemented with N_2 gas had a positive effect on hatchability. Proudfoot (1965) showed that the Cryovac N_2 packing method tended to maintain the O_2 concentration at about 4% and stabilized albumen and yolk pH. Albumen pH was maintained at the fresh egg level. Proudfoot (1965) proposed that a temporary displacement of O_2 by N_2 could result in a more chemical stability of the egg components during storage. He also suggested that high CO_2 concentrations had a severe toxic effect on the embryo. Whether these hypotheses are true, is unknown. It seems that a prevention of gaseous exchange during egg storage has a positive effect on hatchability and that a low O_2 concentration during storage is not detrimental for hatchability. Becker et al. (1968) investigated the effect of high CO_2 concentrations in the albumen at the end of storage (just prior to incubation) 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, which were stored for different durations, were placed in a Cryovac plastic bag. The plastic bag was filled with CO_2 gas for one hour. Due to the CO_2 treatment, the albumen pH decreased almost to the level of a fresh laid egg. The effect on hatchability was not consistent and the average hatchability over all storage durations (from 0 to 21 days) indicated that there was no difference between the controls and the treated eggs in hatchability of fertile eggs (on average 79.1% vs. 78.8%, respectively). After 24 hours of incubation, the albumen pH of treated and control eggs were almost similar (9.201 vs. 9.296, respectively). During storage, the albumen pH was also comparable for the treated and control eggs. The only difference was seen in the pH levels at the onset of the incubation process. Becker et al. (1968), therefore, suggested that the

high level of albumen pH during storage negatively affected embryo viability after prolonged egg storage. On the other hand, it may be possible that the increase in albumen pH during the first 2 days of incubation that always occurs, negatively affected embryonic development.

Embryo and Egg Characteristics during Early Incubation

The first part of the incubation process is the developmental phase. In the developmental phase, the embryo forms from the area pellucida and the following membranes and fluid compartments form 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 (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 break down 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 may be caused by a delay in embryonic development caused by prolonged egg storage. This could lengthen the time that embryos are exposed to the alkaline albumen, because amnion development is retarded due to prolonged egg storage. Consequently, the embryo may 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 onset of incubation. Published work has shown that delays occur in embryonic development after prolonged egg storage (Kaufman, 1938; Steinke, 1972). Kaufman (1938) found, based on the stage of embryonic development, that embryos from eggs stored between 24 days 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.

A delay in embryonic development after prolonged egg storage durations may be reduced when eggs are prewarmed fast from storage temperature to the optimal eggshell temperature of 37.8°C (Lourens et al., 2005) at the onset of incubation. Little information is available in the literature regarding the effect of the rate of preincubation warming on hatchability and chick quality. Meijerhof et al. (1994) investigated the effect of presetting temperatures on hatchability of fertile eggs, which were 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. For the young flock, hatchability of fertile eggs did not differ. However, for the old flock, hatchability of fertile eggs was significantly higher in the preincubation warming temperature treatment of 20°C (89.0%) compared to 27°C (85.1%). As stated earlier, embryos of older breeders seem to be more sensitive to temperatures above 20°C than embryos of young breeders (Meijerhof et al., 1994). Abnormal development usually occurs at temperatures between 27°C to 35°C (Wilson, 1991). This may explain the negative effect of a preincubation warming period of 16 hours at a temperature of 27°C on hatchability in the older breeder flock. However, the hatchability difference found between eggs of young and old breeders is difficult to explain. Meijerhof et al. (1994) speculated that the reduced fertility and hatchability found when bird age increases may 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 affect embryo viability and, therefore, the ability of the embryo to endure sub-optimal storage conditions. In addition, it may be more difficult for the embryo to regulate the pH of its micro-environment when albumen and eggshell quality decrease when the age of the breeder flock increases. A decrease in albumen and eggshell quality increases CO_2 loss and consequently increases albumen pH.

Mayes and Takeballi (1984) reviewed the results of other studies that examined the rate of preincubation warming just prior to setting. Most authors cited by Mayes and Takeballi (1984) 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 these studies reviewed by Mayes and Takeballi (1984), preincubation warming lasted for more than 16 hours. No information in the published literature can be found regarding the effect of a fast preincubation warming profile on hatchability and chick quality. It can be hypothesized that when the preincubation warming profile is fast, eggs are exposed to sub-optimal incubation temperatures for a short period of time. When the preincubation warming profile is slow (12 to 24 hours), mitosis occurs at sub-optimal temperatures for a certain period of time, and abnormal development or a delay in embryonic development may occur. Another possibility is that the mitotic rate is low and consequently, less viable embryonic

cells are available to form an effective barrier between the inside of the embryo and its exterior or to produce sufficient CO_2 to reduce the pH to a level that ranges from 7.9 to 8.4 in the micro-environment of the embryo during the first few days of incubation. The negative effects of a slow preincubation warming profile on embryonic development may be enhanced in long-term stored embryos because long-term stored embryos probably contain less viable embryonic cells than short-term stored embryos. To investigate whether these hypotheses are true, more research is needed.

When the embryo is unable to regulate the pH in its micro-environment during early incubation, high CO_2 concentrations in the incubator during early incubation may help to optimize the pH in the micro-environment of the embryo. If the CO_2 pressure outside the egg is higher than inside the egg, the loss of CO_2 from the eggs halts and the albumen pH may decrease as a result of the CO_2 production of the embryo. Whether high CO_2 concentrations in the incubator during early incubation are positive for embryonic development has been reviewed by Onagbesan et al. (2007) and is an important topic for research (De Smit et al., 2006; Bruggeman et al., 2007).

Conclusions

It can be concluded that during storage, cell death increases or embryo viability is negatively affected, or both. The reason for this is unknown, but the storage duration and changes in the egg characteristics may be two contributing factors. Embryo viability seems to depend on the stage of embryonic development, the total number of viable embryonic cells, and the pH of the micro-environment of the embryo. When egg storage is short, the stage of embryonic development is not important, probably because cell death is low. However, during prolonged egg storage, the stage of embryonic development may hold greater importance in maintaining a sufficient population of viable embryonic cells. An embryo in an advanced, but quiescent developmental stage may be able to form an effective barrier against the alkaline albumen and may be able to produce a sufficient amount of CO_2 to reduce the pH in the micro-environment of the embryo 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 unable to regulate this, altering the atmosphere during storage, during early incubation, or both seems to be crucial for the optimal continuation 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 unable to regulate the pH of its micro-environment more research is needed to investigate the optimal atmosphere during storage and early incubation.

Acknowledgments

We are grateful to Dr. Gaylene Fasenko (Department of Agricultural Food and Nutritional Science, University of Alberta, Canada) and Dr. Henny van Straaten (Department of Anatomy and Embryology, Maastricht University, the Netherlands) for reviewing this manuscript and give very useful comments.

Chapter 2

Influence of Prestorage Incubation on Embryonic Development, Hatchability, and Chick Quality

I.A.M. Reijrink[1], R. Meijerhof[1], B. Kemp[2], E.A.M. Graat[3], and H. van den Brand[2]

[1]HatchTech Incubation Technology B.V., P.O. Box 256, 3900 AG Veenendaal, the Netherlands; [2]Adaptation Physiology Group and [3]Quantitative Veterinary Epidemiology Group, Wageningen Institute of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands.

Reprinted by the permission of the Poultry Science Association
Poultry Science (2009) 88:2649-2660

Abstract

Egg storage beyond 7 days is associated with a delay in hatch time and a decline in hatchability and chick quality. Prestorage incubation is suggested as a method to reduce the negative effects of prolonged egg storage by altering the stage of embryonic development, but earlier research has shown that prestorage incubation can both be detrimental and beneficial for hatchability. The reason for these ambiguous results is unclear and the effect of prestorage incubation on chick quality is not studied extensively. The objective of the current study was to investigate changes in the stage of embryonic development during prestorage incubation and the effect of prestorage incubation on hatchability and chick quality.

Two experiments were conducted. In experiment I, eggs were stored for 3, 5, 8, or 12 days. In experiment II, eggs were stored for 5 or 11 days. Half of the eggs were stored immediately at 16°C to 18°C and the other half were exposed to prestorage incubation for 6 hours in experiment I and for 4.5 hours in experiment II. According to the classification table of Eyal-Giladi and Kochav (EG), the stage of embryonic development advanced by prestorage incubation from EG11.67 to EG13.26 in experiment I ($P = 0.02$) and from EG9.22 to EG12.63 in experiment II ($P < 0.001$). In experiment I, prestorage incubation reduced hatchability of set eggs from 59.3 to 51.5% when storage duration was 12 days, but did not reduce hatchability when storage duration was 3, 5, or 8 days (interaction $P = 0.02$). Prestorage incubation increased chick length ($P = 0.004$). In experiment II, prestorage incubation increased hatchability of fertile eggs from 80.6 to 85.9% when storage duration was 11 days, but did not increase hatchability when storage duration was 5 days (interaction $P < 0.001$). Prestorage incubation increased percentage of second-grade chicks ($P = 0.01$). It seems that storage duration, embryonic development at egg collection, and prestorage incubation duration determine the effect of prestorage incubation on hatchability and chick quality.

Key words: prestorage incubation, storage duration, embryonic development, hatchability, chick quality

Introduction

Due to the variable market demand for day-old chicks in the poultry industry and maximum hatchery capacity, duration of egg storage varies between a few days and several weeks. Egg storage beyond 7 days is associated with a delay in hatch time (Mather and Laughlin, 1976; Tona et al., 2003), a decline in hatchability (Becker, 1964; Merritt, 1964; Fasenko et al., 2001b; Tona et al., 2004; Yassin et al., 2008), and a decline in chick quality (Byng and Nash, 1962; Merritt, 1964; Tona et al., 2003, 2004). Several authors investigated the effect of prestorage incubation to reduce the negative effects of prolonged egg storage on hatchability (Becker and Bearse, 1958; Bowling and Howarth, 1981; Proudfoot and Hulan, 1982; Meir and Ar, 1998; Fasenko et al., 2001a, b). In general, it can be concluded that prestorage incubation has no effect on hatchability when storage duration is shorter than 7 days, and can both be detrimental and beneficial when storage duration is beyond 7 days. The effect of prestorage incubation on chick quality is not studied extensively.

According to Kosin (1956) and Fasenko et al. (2001b), the effect of prestorage incubation on hatchability depends on the stage of embryonic development after prestorage incubation. Fasenko et al. (2001b) hypothesized that embryos at developmental stage EG13 (according to the classification table of Eyal-Giladi and Kochav, 1976 (EG)) are more resistant to prolonged egg storage than embryos that are less or further advanced than stage EG13. At this developmental stage, the embryo has completed hypoblast formation, and cell migration and differentiation is minimal (Bellairs, 1986). These embryos, therefore, contain more cells than embryos that are less-advanced than stage EG13 and are in a more quiescent stage of embryonic development than embryos that are further advanced than stage EG13, which probably make them more resistant to prolonged egg storage. In embryos that are less or further advanced than stage EG13, damage caused by prolonged egg storage may be irreversible and may cause embryonic mortality. Fasenko et al. (2001b) hypothesized that there is one optimal stage of development to maintain embryo viability during prolonged egg storage, but there can also be an optimal range of stages. The objective of the current study was to investigate the effect of prestorage incubation on hatchability and also on chick quality and to investigate whether results of the experiments could be explained by the stages of embryonic development at egg collection, or after prestorage incubation, or both.

Materials and Methods

Experimental Setup

Two experiments were performed. Experiment I was a 4 x 2 factorial design: 4 storage durations (3, 5, 8, or 12 days) and 2 prestorage incubation durations (0 or 6 hours). Experiment II was a 2 x 2 factorial design: 2 storage durations (5 or 11 days) and 2 prestorage incubation

durations (0 or 4.5 hours). Both experiments were conducted under practical circumstances in a commercial hatchery.

Breeder Flock

For the two experiments, eggs from two different commercial Cobb broiler breeder flocks were used. At the start of experiment I, breeder flock age was 61 weeks, whereas at the start of experiment II, breeder flock age was 28 weeks. In both experiments, broiler breeders were housed in 60 cages, with in each cage on average 455 females and 39 males. Hens were restrictedly fed according to the Cobb guideline. Water was available 4 hours each day.

Egg Collection, Prestorage Incubation, and Storage

The day before the start of the experiment, the last egg collection was at 1430 h. Lights were out between 1900 h and 0400 h. Eggs were collected between 4 hours and 6 hours after lights were switched on. In experiment I, a total of 9,652 eggs were collected on day 3, 5, 8, and 12 before setting. In experiment II, a total of 4,826 were collected on day 5 and 11 before setting.

Egg collection for experiment I was conducted when outside temperature was around 28°C during the day. For experiment II, this was around 18°C. At each collection day, half of the eggs were exposed to prestorage incubation (treated eggs) and half of the eggs were stored immediately in a storage room at the breeder farm (control eggs). Temperature in the storage room was maintained at 16°C to 18°C. Relative humidity was not measured and uncontrolled. During prestorage incubation, internal egg temperature was measured in three eggs per collection day. A sensor (NTC Thermistors: type DC 95,Thermometrics, Somerset, UK) was inserted in the egg for 15 mm through a hole of 3 mm in diameter in the eggshell in the middle of the blunt end of the egg. Treated eggs were warmed in an incubator to an internal egg temperature of 37.8°C within 1.5 hours and this internal egg temperature was maintained for 4.5 hours in experiment I (treatment 6 hours) and for 3 hours in experiment II (treatment 4.5 hours). Thereafter, eggs were cooled down in the incubator for 3 hours to an internal temperature of 24°C. During prestorage incubation, relative humidity varied between 45% and 50% and CO_2 concentration was 0.02%. After prestorage incubation, eggs used to measure internal egg temperatures were removed from the experiment and all other eggs were stored in the same storage room as the control eggs. The duration of prestorage incubation was reduced from 6 hours in experiment I to 4.5 hours in experiment II, because in experiment I, hatchability of fertile eggs was not improved by prestorage incubation.

To calculate egg weight loss, 64 eggs per treatment group (8 eggs per setter tray) were individually weighed on day of oviposition and the same 64 eggs were weighed on the last day of storage and on day 18 of incubation. Egg weight loss during storage was determined

by calculating the difference in egg weight between oviposition and last day of storage as a percentage of egg weight at oviposition; egg weight loss during incubation was determined by calculating the difference in egg weight between the last day of storage and day 18 of incubation as a percentage of egg weight on the last day of storage. Total egg weight loss was the sum of the egg weight loss during storage and incubation. In experiment I, 20 control eggs and 20 treated eggs were opened the day after oviposition to examine the stage of embryonic development. In experiment II, the same examination was done in 10 control and 10 treated eggs. The filter ring technique, as described by Gupta and Bakst (1993), was used to isolate the embryo from the yolk membrane. After isolation, the embryo was flushed with buffered saline to remove yolk residue. The dorsal and ventral side of the embryo were examined with a stereo microscope (Olympus SZ61, Olympus Nederland B.V., Zoeterwoude, the Netherlands) to determine the stage of embryonic development according to the classification table of Eyal-Giladi and Kochav (1976) (**EG**) or Hamburger and Hamilton (1951) (**HH**). The classification table of Eyal-Giladi and Kochav (1976) measures embryonic development on the basis of morphogenetic movements on the surface of the embryo (Fasenko et al., 1992a). The classification table of Hamburger and Hamilton (1951) measures embryonic development on the basis of external factors, such as the number of somite pairs, changes in the wings, legs, visceral arches, feather germs, and eyelids; and the length of the beak and toes.

Incubation

Before setting, all eggs were disinfected for 20 minutes with 7 g/m^3 paraformaldehyde without increasing the temperature. After 20 minutes of disinfection, the disinfection room was ventilated with fresh air for 1.5 hours to remove the paraformaldehyde residues. Afterward, eggs were set in an incubator (HT-57,600, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands). Per treatment (storage duration x prestorage incubation duration), 8 setter trays were completely filled with 150 eggs and were distributed in two setter trolleys. Eggshell temperature was maintained at 37.8°C throughout incubation (Lourens et al., 2005) and relative humidity varied between 60% and 80% during the first 4 days of incubation and between 35% and 45% afterward. The inlet and outlet valve of the setter and hatcher were controlled to maintain the CO_2 concentrations below 0.35%. Eggs were turned every hour over 90° until day 18 of incubation.

On day 18 of incubation, eggs were candled and clear eggs were opened to determine macroscopically infertility or stage of embryonic mortality. Eggs that contained living embryos were transferred to hatcher baskets and placed in a hatcher (HT-28,800, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands). In experiment I, eggs that were weighed during the experiment and contained a living embryo on day 18 of incubation, were placed per treatment in a hatcher basket to measure chick length, chick body weight, and navel quality of

the hatched chicks on day 21 of incubation. In experiment II, out of every hatcher basket, 8 to 10 chicks were taken at random on day 21 of incubation to measure chick length, chick body weight and navel quality. In total, chick quality was determined of 65 to 81 chicks per treatment in experiment II. To determine chick length, the chick was laid on its ventral side, with neck and right leg extended to their maximum length. Chick length was defined as the length from the tip of the beak to the implantation of the nail on the middle toe (Hill, 2001; Willemsen et al., 2008). Chick length depends on incubation conditions and seems to be correlated with first week mortality and chick performance posthatch (Hill, 2001; Molenaar et al., 2008). Therefore, chick length was used as a parameter to monitor chick quality in these experiments. Navel quality was analyzed and given a score of 1 if the navel was completely closed and clean; a score of 2 if the navel was discolored (color different from skin color) or opened to a maximum of 2 mm, or both; and a score of 3 was given if the navel was discolored or opened more than 2 mm, or both. All chicks were classified as first- or second-grade chicks based on physical parameters. A chick was classified as a first-grade chick if the chick was clean, dry, free of deformities or lesions, had bright eyes (Tona et al., 2004), and if the chick was given a navel score of 1 or 2. The other chicks were classified as second-grade chicks. On day 21 of incubation, un-hatched eggs were opened to determine stage of embryonic mortality. On day 18 (candling) and 21 of incubation the following stages of embryonic mortality were used to classify the dead embryos: days 1 to 2 (white membrane over the yolk), day 3 (blood ring), days 4 to 9 (black eye visible, embryo without down), days 10 to 17 (small embryo with down), days 18 to 19 (full grown embryo with yolk out), days 20 to 21 (full grown embryo death or alive with yolk subtracted). Fertility was calculated as a percentage of set eggs. Hatchability (first- and second-grade chicks) was calculated as a percentage of set eggs and as a percentage of fertile eggs. Embryonic mortality, cracked eggs and rotten eggs were calculated as a percentage of fertile eggs. Second-grade chicks was calculated as a percentage of total hatched chicks.

Statistical Analysis

Data were analyzed as a 4 x 2 factorial design (experiment I) or 2 x 2 factorial design (experiment II). Fertility (yes-no), embryonic mortality (yes-no), and hatchability of set and fertile eggs (yes-no) were analyzed using logistic regression analysis (PROC LOGISTIC, SAS Institute Inc., 2004) with storage duration, prestorage incubation, and their interaction as class variables. Nonparametric estimates of the survivor function were calculated (PROC LIFETEST, SAS Institute Inc., 2004) and proportional hazard regression (PROC PHREG, SAS Institute Inc., 2004) was performed to compare the survival of embryos per storage duration, prestorage incubation and their interaction. Egg weight, egg weight loss, chick length, chick body weight, and navel quality were analyzed using general linear regression (PROC GLM, SAS Institute

Inc., 2004) with storage duration, prestorage incubation and their interaction as class variables. Unfertilized eggs and eggs that contained dead embryos were excluded from the data when egg weight and egg weight loss were analyzed. Model assumptions were checked by examining the distributions of residuals. Least square means were compared using Bonferroni adjustment for multiple comparisons. Values are expressed as least squares means ± SEM. All main factors and interactions were analyzed for significance at $P \leq 0.05$. Because it was unknown which chick hatched from which egg, chick length and chick body weight were corrected for the average initial egg weight by creating two new variables: chick length/average initial egg weight per treatment and chick body weight/average initial egg weight per treatment. Effect of treatment on stage of embryonic development was compared using the Kruskal-Wallis nonparametric test (PROC NPAR1WAY, SAS Institute Inc., 2004).

Results

Experiment I

Egg Weight and Egg Weight Loss

Initial egg weights were not different among the four collection days and averaged 70.04 g. No interaction was found between storage duration and prestorage incubation for egg weight loss during storage, incubation, and total egg weight loss (Table 1). Percentage of egg weight loss during storage increased when storage duration increased ($P < 0.001$). Storage duration did not affect percentage of egg weight loss during incubation. Total egg weight loss increased when storage duration increased ($P = 0.001$) as a consequence of the difference in egg weight loss during storage. Prestorage incubation increased egg weight loss during storage by 0.18% ($P < 0.001$). Prestorage incubation did not affect egg weight loss during incubation and total egg weight loss.

Table 1. Effect of storage duration and prestorage incubation on egg weight (loss) (experiment I)

Treatment	n	Fresh egg weight, g	Egg weight loss during storage, %	Egg weight loss during incubation, %	Total egg weight loss, %
Storage duration, d					
3	82	70.30	0.24^{d}	10.07	10.29^{b}
5	81	70.06	0.53^{c}	9.48	9.95^{b}
8	72	70.10	0.74^{b}	9.78	10.44^{ab}
12	61	69.71	1.28^{a}	9.95	11.10^{a}
SEM		0.51	0.01	0.19	0.20
Prestorage incubation, h					
0	146	69.95	0.61^{b}	9.85	10.39
6	150	70.13	0.79^{a}	9.79	10.50
SEM		0.36	0.01	0.14	0.14
Storage duration, d x prestorage incubation, h					
3 x 0	38	69.98	0.15	10.45	10.58
3 x 6	44	70.62	0.33	9.69	9.99
5 x 0	40	70.78	0.43	9.64	10.02
5 x 6	41	69.33	0.63	9.32	9.88
8 x 0	39	70.43	0.64	9.47	10.05
8 x 6	33	69.76	0.84	10.08	10.84
12 x 0	29	68.71	1.21	9.84	10.93
12 x 6	32	70.82	1.35	10.06	11.27
SEM		0.72	0.02	0.28	0.29
***P*-value**					
Storage duration		0.88	<0.001	0.13	0.001
Prestorage incubation		0.72	<0.001	0.76	0.62
Interaction		0.07	0.48	0.06	0.08

$^{a\text{-}d}$Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$).

Embryonic Development

The developmental stage of the control embryos varied from EG10 to HH3 (Figure 1). Twenty percent of the control embryos was advanced beyond developmental stage EG13. The stage of embryonic development was advanced from EG11.67 to EG13.26 by prestorage incubation

($P = 0.02$). The developmental stage of the treated embryos varied from EG10 to HH3+. Fifty eight percent of the treated embryos was advanced beyond developmental stage EG13.

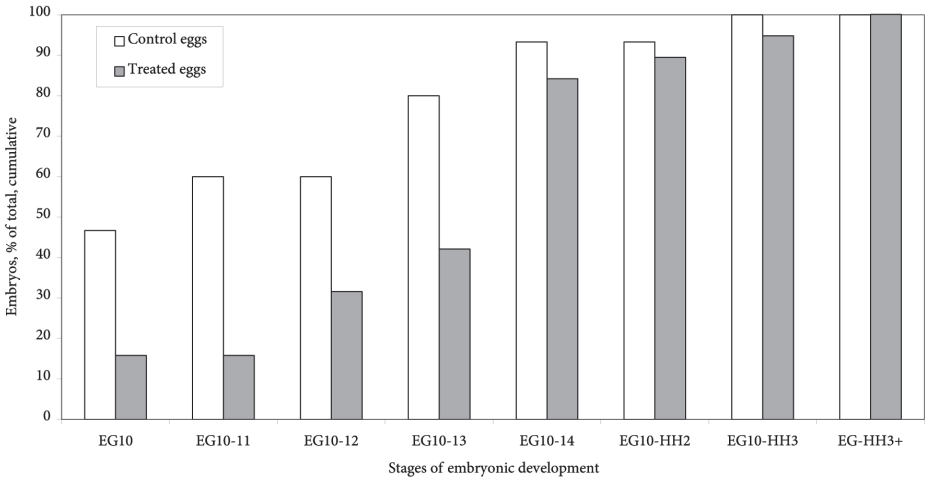

Figure 1. Cumulative percentage of total number of embryos in particular stages of embryonic development in control and treated eggs (Experiment I; n = 40). EG = the stage of embryonic development according to Eyal-Giladi and Kochav (1976); HH = the stage of embryonic development according to Hamburger and Hamilton (1951).

Fertility, Embryonic Mortality, and Hatchability

Hatchability of set eggs was 7.8% lower for treated eggs than for control eggs when storage duration was 12 days. Within the other storage durations, prestorage incubation had no effect on hatchability of set eggs (interaction $P = 0.02$; Table 2). Embryonic mortality from days 1 to 2 of incubation was 2.7% higher in treated eggs than in control eggs when storage duration was 12 days. Within the other storage durations, prestorage incubation had no effect on embryonic mortality from days 1 to 2 of incubation (interaction $P = 0.04$). Hatchability of fertile eggs stored for 12 days was lower than hatchability of fertile eggs stored for 3, 5, and 8 days ($P = 0.005$). Proportional hazard regression also showed that storage for 12 days increased the hazard of embryonic mortality in comparison to 3, 5, and 8 days ($P < 0.001$; Figure 2). Embryonic mortality from days 18 to 19 of incubation in eggs stored for 12 days was higher than in eggs stored for 3 and 5 days ($P = 0.03$). Prestorage incubation did not affect hatchability of fertile eggs.

Table 2. Effect of storage duration and prestorage incubation on fertility, hatchability, embryonic mortality, and percentage of second-grade chicks (experiment I)

					Embryonic mortality, % of fertile eggs								
Treatment	**n**	**Fertility, %**	**Hatchability of set eggs, %**	**Hatchability of fertile eggs, %**	**d 1 to 2**	**d 3**	**d 4 to 9**	**d 10 to 17**	**d 18 to 19**	**d 20 to 21**	**Cracked eggs[1], %**	**Rotten eggs[1], %**	**Second-grade chicks[2], %**
Storage duration, d													
3	2,400	73.5	61.2	83.4^{a}	5.3	1.2	2.2	1.3	3.3^{b}	1.1	0.3	1.9	3.1
5	2,400	71.5	60.9	85.0^{a}	3.5	1.3	1.9	1.2	2.8^{b}	1.5	0.6	2.2	2.7
8	2,400	72.3	59.8	82.6^{a}	4.1	1.0	2.0	1.9	3.9^{ab}	1.6	0.7	2.0	2.6
12	2,400	71.6	55.4	77.1^{b}	8.7	1.6	2.7	1.2	4.7^{a}	1.6	0.7	1.4	4.2
Prestorage incubation, h													
0	4,800	73.0	60.8	83.4	5.3	1.2	1.9	1.3	3.5	1.5	0.5	1.5	3.4
6	4,800	71.5	57.9	80.7	5.5	1.4	2.5	1.5	3.8	1.4	0.6	2.3	2.8
Storage duration, d x prestorage incubation, h													
3 x 0	1,200	73.4	61.6	84.2	5.8	1.3	1.9	0.9	3.0	0.8	0.2	2.0	3.1
3 x 6	1,200	73.6	60.7	82.6	4.8	1.1	2.5	1.7	3.7	1.4	0.1	2.0	3.0
5 x 0	1,200	72.5	62.7	86.3	3.0	1.4	1.7	1.4	2.3	1.5	0.7	1.8	3.2
5 x 6	1,200	70.5	59.2	83.7	4.0	1.3	2.0	0.9	3.3	1.5	0.5	2.7	2.3
8 x 0	1,200	71.8	59.6	82.9	4.9	0.9	1.3	1.3	4.8	1.5	0.8	1.7	2.6
8 x 6	1,200	72.8	60.1	82.2	3.4	1.2	2.6	2.5	3.1	1.7	0.7	2.4	2.6
12 x 0	1,200	74.1	59.3^{a}	80.1	7.4^{b}	1.1	2.6	1.5	4.2	2.1	0.2	0.7	4.9
12 x 6	1,200	69.1	51.5^{b}	73.9	10.1^{a}	2.2	3.0	1.0	5.2	1.0	1.2	2.2	3.4
***P*-value**													
Storage duration		0.62	0.27	0.005	<0.001	0.84	0.25	0.74	0.03	0.16	0.21	0.16	0.08
Prestorage incubation		0.93	0.59	0.35	0.33	0.82	0.42	0.15	0.36	0.26	0.57	0.99	0.93
Interaction		0.10	0.02	0.33	0.04	0.53	0.61	0.10	0.10	0.17	0.15	0.25	0.68

$^{a-b}$Means of the main effects within a column and factor lacking a common superscript differ ($P \leq 0.05$). $^{a-b}$Means of the interaction within one storage duration lacking a common superscript differ ($P \leq 0.05$). [1]Number of cracked and rotten eggs as a percentage of the number of fertile eggs. [2]Number of second-grade chicks as a percentage of total number of hatched chicks.

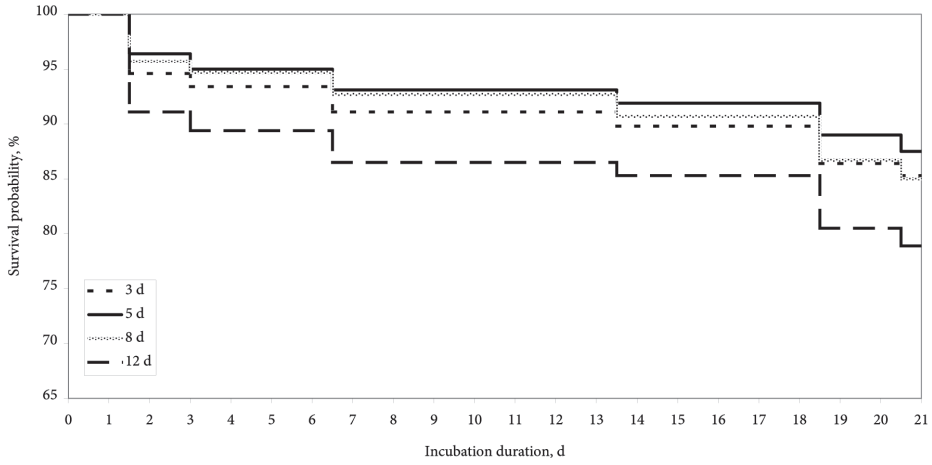

Figure 2. Embryo survival curves for four different storage durations (3, 5, 8, and 12 days; Experiment I). Embryos died either between days 1 to 2 (1.5), on day 3 (3), between days 4 to 9 (6.5), between days 10 to 17 (13.5), between days 18 to 19 (18.5), or between days 20 to 21 of incubation (20.5).

Chick Quality

No interaction was found between storage duration and prestorage incubation for chick length corrected for egg weight, chick body weight corrected for egg weight, navel quality (Table 3), and percentage of second-grade chicks (Table 2). Chick length corrected for egg weight was higher for eggs stored for 3 days than for eggs stored for 5, 8, and 12 days ($P < 0.001$). Storage duration did not affect chick body weight corrected for egg weight and navel quality. Prestorage incubation increased chick length corrected for egg weight with 0.003 cm/g ($P = 0.004$). Prestorage incubation did not affect chick body weight corrected for egg weight and navel quality. Storage duration and prestorage incubation did not affect percentage of second-grade chicks.

Table 3. Effect of storage duration and prestorage incubation on chick length, chick body weight, and navel quality (experiment I)

Treatment	n	Chick length/ average initial egg weight, cm/g	Chick length,[1] cm	Chick body weight/average initial egg weight, g/g	Chick body weight, g	Navel quality[2]
Storage duration, d						
3	75	0.295[a]	20.6	0.670	46.8	1.49
5	79	0.291[b]	20.4	0.666	46.8	1.44
8	72	0.291[b]	20.3	0.677	47.4	1.50
12	60	0.289[b]	20.3	0.676	47.6	1.55
SEM		0.001		0.006		0.06
Prestorage incubation, h						
0	141	0.290[b]	20.4	0.677	47.6	1.50
6	145	0.293[a]	20.5	0.668	46.7	1.49
SEM		0.001		0.004		0.04
Storage duration, d x Prestorage incubation, h						
3 x 0	36	0.296	20.6	0.672	46.7	1.53
3 x 6	39	0.295	20.7	0.668	46.8	1.46
5 x 0	40	0.289	20.5	0.666	47.4	1.45
5 x 6	39	0.293	20.3	0.665	46.2	1.44
8 x 0	39	0.288	20.2	0.690	48.4	1.49
8 x 6	33	0.294	20.5	0.664	46.2	1.52
12 x 0	30	0.288	20.1	0.680	47.6	1.53
12 x 6	30	0.290	20.5	0.673	47.5	1.57
SEM		0.001		0.008		0.09
***P*-value**						
Storage duration		<0.001		0.47		0.70
Prestorage incubation		0.004		0.10		0.94
Interaction		0.06		0.39		0.94

[a-b]Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$). [1]The values for chick length and chick body weight are least squares means, but are not analyzed for significant differences because the values have to be corrected for initial egg weight. [2]Mean of navel score 1 to 3.

Experiment II

Egg Weight and Egg Weight Loss

Initial egg weights were different between the two collection days (P = 0.006) (Table 4). Prestorage incubation did not affect percentage of egg weight loss during storage when storage duration was 5 days, but increased egg weight loss by 0.16% when storage duration was 11 days (interaction P = 0.02). No interaction was found between storage duration and prestorage incubation for egg weight loss during incubation, and total egg weight loss. Storage duration did not affect percentage of egg weight loss during incubation. Total egg weight loss increased when storage duration increased (P < 0.001) as a consequence of the difference in egg weight loss during storage. Prestorage incubation did not affect egg weight loss during incubation and total egg weight loss.

Table 4. Effect of storage duration and prestorage incubation on egg weight (loss) (experiment II)

Treatment	n	Fresh egg weight, g	Egg weight loss during storage, %	Egg weight loss during incubation, %	Total egg weight loss, %
Storage duration, d					
5	117	58.15[a]	0.70	8.50	9.14[b]
11	103	56.78[b]	1.43	8.55	9.86[a]
SEM		0.34	0.02	0.13	0.14
Prestorage incubation, h					
0	116	57.47	1.01	8.56	9.48
4.5	104	57.46	1.12	8.50	9.52
SEM		0.34	0.02	0.13	0.14
Storage duration, d x prestorage incubation, h					
5 x 0	62	57.88	0.67[c]	8.68	9.29
5 x 4.5	55	58.42	0.73[c]	8.33	9.00
11 x 0	54	57.06	1.35[b]	8.44	9.67
11 x 4.5	49	56.50	1.51[a]	8.66	10.04
SEM		0.48	0.02	0.18	0.20
***P*-value**					
Storage duration		0.006	<0.001	0.80	<0.001
Prestorage incubation		0.96	<0.001	0.75	0.83
Interaction		0.25	0.02	0.11	0.09

[a-c]Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$).

Embryonic Development

The developmental stage of the control embryos varied from EG8 to EG10 (Figure 3). Seventy-five percent of the control embryos was in a developmental stage below EG10. The stage of embryonic development was advanced from EG9.22 to EG12.63 by prestorage incubation ($P < 0.001$). The developmental stage of the treated embryos varied from EG11 to HH3+. Forty percent of the treated embryos was advanced beyond developmental stage EG13.

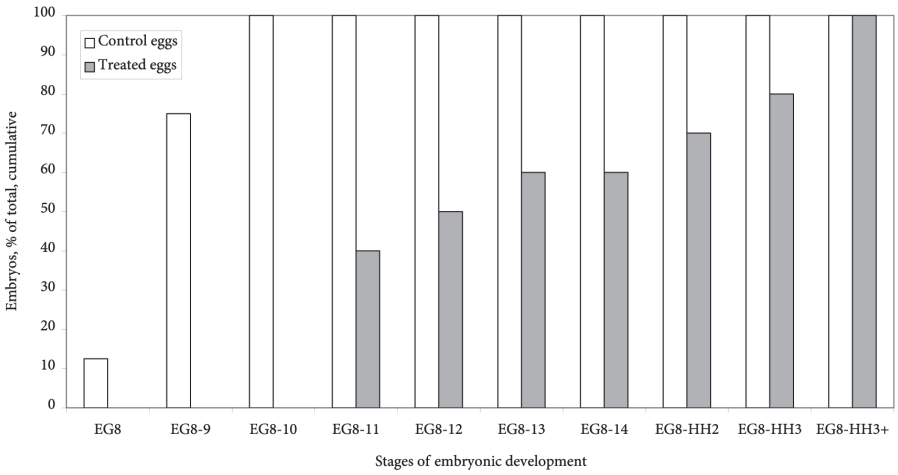

Figure 3. Cumulative percentage of total number of embryos in particular stages of embryonic development in control and treated eggs (experiment II; n = 20). EG = the stage of embryonic development according to Eyal-Giladi and Kochav (1976); HH = the stage of embryonic development according to Hamburger and Hamilton (1951).

Fertility, Embryonic Mortality, and Hatchability

Hatchability of set and fertile eggs was 5.4 and 5.3% higher, respectively for treated eggs than for control eggs when storage duration was 11 days. Within eggs stored for 5 days, prestorage incubation had no effect on hatchability of set and fertile eggs (interaction $P = 0.001$ and $P < 0.001$, respectively; Table 5). The difference in hatchability when storage duration was 11 days, was caused by a higher percentage (3.1%) of embryonic mortality during the first 2 days of incubation in the control group compared to the treated group (interaction $P = 0.03$).

Proportional hazard regression also showed this interaction between storage duration and prestorage incubation for the hazard of embryonic mortality during incubation ($P < 0.001$) (Figure 4). Storage duration did not affect embryonic mortality after day 9 of incubation. Prestorage incubation increased embryonic mortality from days 18 to 19 of incubation by 1.7% ($P = 0.003$).

Table 5. Effect of storage duration and prestorage incubation on fertility, hatchability, embryonic mortality, and percentage of second-grade chicks (experiment II)

					Embryonic mortality, % of fertile eggs							
Treatment	**n**	**Fertility, %**	**Hatchability of set eggs, %**	**Hatchability of fertile eggs, %**	**d 1 to 2**	**d 3**	**d 4 to 9**	**d 10 to 17**	**d 18 to 19**	**d 20 to 21**	**Cracked eggs[1], %**	**Second-grade chicks[2], %**
Storage duration, d												
5	2,400	96.1	84.8	88.2	3.4	1.3[b]	1.7[b]	0.9	2.6	1.6	0.3	1.7
11	2,400	95.7	79.9	83.3	5.7	2.8[a]	2.7[a]	0.4	2.0	2.3	0.7	1.7
Prestorage incubation, h												
0	2,400	95.9	81.4	84.9	5.3	2.3	2.5	0.8	1.2[b]	1.8	0.6	1.0[b]
4.5	2,400	96.0	83.1	86.6	3.8	1.8	1.9	0.5	2.9[a]	2.1	0.3	2.4[a]
Storage duration, d x prestorage incubation, h												
5 x 0	1,200	96.3	85.8	89.2	3.4	1.2	1.7	1.0	1.6	1.4	0.5	1.0
5 x 4.5	1,200	96.0	83.8	87.2	3.5	1.5	1.6	0.8	3.6	1.8	0.0	2.5
11 x 0	1,200	95.5	77.0[b]	80.6[b]	7.3[a]	3.5	3.3	0.6	1.7	2.2	0.6	1.0
11 x 4.5	1,200	95.9	82.4[a]	85.9[a]	4.2[b]	2.2	2.2	0.3	2.2	2.4	0.7	2.3
***P*-value**												
Storage duration		0.47	<0.001	<0.001	<0.001	<0.001	0.02	0.36	0.73	0.15	0.77	0.99
Prestorage incubation		0.88	0.16	0.15	0.90	0.58	0.88	0.67	0.003	0.40	0.95	0.01
Interaction		0.56	0.001	<0.001	0.03	0.12	0.35	0.43	0.13	0.70	0.94	0.89

[a-b]Means of the main effects within a column and factor lacking a common superscript differ ($P \leq 0.05$). [a-b]Means of the interaction within one storage duration lacking a common superscript differ ($P \leq 0.05$). [1]Number of cracked eggs as a percentage of the number of fertile eggs. [2]Number of second-grade chicks as a percentage of total number of hatched chicks.

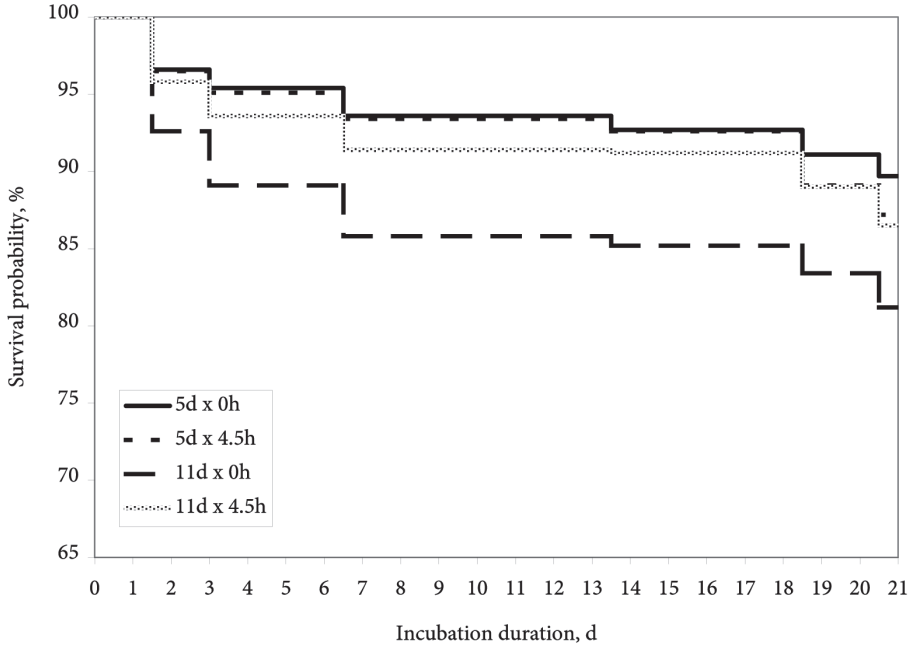

Figure 4. Embryo survival curves for the interaction between storage duration (5 and 11 days) and prestorage incubation (0 hours and 4.5 hours; Experiment II). Embryos died either between days 1 to 2 (1.5), on day 3 (3), between days 4 to 9 (6.5), between days 10 to 17 (13.5), between days 18 to 19 (18.5), or between days 20 to 21 of incubation (20.5).

Chick Quality

Chick length corrected for egg weight of the control and treated group was not different when storage duration was 11 days, whereas it was 0.006 cm/g higher for the control group than for the treated group when storage duration was 5 days (interaction $P = 0.02$; Table 6). Chick body weight corrected for egg weight of the control and treated group was not different when storage duration was 5 days, whereas it was 0.026 g/g higher for the control group than for the treated group when storage duration was 11 days (interaction $P = 0.03$). No interaction was found between storage duration and prestorage incubation for navel quality. Storage duration did not affect percentage of second-grade chicks (Table 5). Storage duration reduced navel quality ($P = 0.03$; Table 6). Prestorage incubation increased the percentage of second-grade chicks from 1.0 to 2.4% ($P = 0.01$; Table 5). Prestorage incubation did not affect navel quality.

Table 6. Effect of storage duration and prestorage incubation on chick length, chick body weight, and navel quality (experiment II)

Treatment	n	Chick length/ average initial egg weight, cm/g	Chick length[1], cm	Chick body weight/ average initial egg weight, g/g	Chick body weight, g	Navel quality[2]
Storage duration, d						
5	148	0.338	19.6	0.672	39.0	1.53[b]
11	138	0.340	19.3	0.686	38.9	1.67[a]
SEM		0.001		0.004		0.05
Prestorage incubation, h						
0	131	0.341	19.5	0.686	39.3	1.64
4.5	155	0.337	19.4	0.673	38.6	1.56
SEM		0.001		0.004		0.05
Storage duration, d x Prestorage incubation, h						
5 x 0	67	0.341[a]	19.7	0.673[b]	38.9	1.52
5 x 4.5	81	0.335[b]	19.5	0.672[b]	39.1	1.53
11 x 0	64	0.340[a]	19.3	0.699[a]	39.7	1.75
11 x 4.5	74	0.340[a]	19.3	0.673[b]	38.2	1.59
SEM		0.001		0.006		0.07
***P*-value**						
Storage duration		0.05		0.02		0.03
Prestorage incubation		0.002		0.02		0.28
Interaction		0.02		0.03		0.23

[a-b]Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$). [1]The values for chick length and chick body weight are least squares means, but are not analyzed for significant differences because the values have to be corrected for initial egg weight. [2]Mean of navel score 1 to 3.

Discussion

The current study showed that prestorage incubation can have a negative as well as a positive effect on hatchability and chick quality. Both negative and positive effects of prestorage incubation

on hatchability were mainly caused by an increase or decrease in embryonic mortality during the first 2 days of incubation. This suggests that prestorage incubation affects embryo viability and, therefore, survival rate of the embryo during early incubation. Fasenko et al. (2001b) hypothesized that the optimal stage of embryonic development to resist prolonged egg storage is EG13. Embryos in this stage of development completely formed the hypoblast and are in a quiescent stage of embryonic development. Whereas, embryos less developed than stage EG13 are less differentiated and contain less cells, and embryos further developed than EG13 are in a more active stage of embryonic development. The hypothesis of Fasenko et al. (2001b) was that prestorage incubation can be used to bring embryos in the optimal stage of development to resist prolonged egg storage, but prestorage can also be too long. In both experiments of the current study, although a relative high percentage of embryos were further developed than stage EG13 after prestorage incubation, both a positive and negative effect of prestorage incubation on hatchability was found. In experiment I, 58% of the embryos were further developed than stage EG13 after prestorage incubation of 6 hours. In experiment II, 40% of the embryos were further developed than stage EG13 after prestorage incubation of 4.5 hours. Consequently, the difference in the effect of prestorage incubation on hatchability between the two experiments may not be explained by the percentage of embryos that were further developed than stage EG13. More reasonable, the difference in the effect of prestorage incubation on hatchability may be explained by the difference in the stages of embryonic development at egg collection (the control embryos in the current study). In experiment II, 75% of the control embryos were in a developmental stage below EG10. However, in experiment I, all embryos were at developmental stage EG10 or even further advanced. It can be hypothesized that prestorage incubation is most beneficial when embryos are less-developed than stage EG10 at egg collection, which was also stated by Meir and Ar (1998). It is reported that the stage of embryonic development at oviposition is related to hatchability (Hays and Nicolaides, 1934; Coleman and Siegel, 1966; Kosin and Arora, 1966; Steinke, 1972). Steinke (1972) showed that embryos at a developmental stage below EG10 were common in eggs of hens with a hatchability lower than 55%, while eggs of hens with moderate and very good hatchability contained embryos at the developmental stage EG10 or further advanced. The stage of embryonic development at the onset of prestorage incubation was different between the two experiments in the current study. Control embryos of experiment I probably did not need prestorage incubation because the average stage of embryonic development was around EG13 at egg collection. However, control embryos of experiment II probably needed prestorage incubation to bring them in a developmental stage equal to or further advanced than EG10.

One of the factors that may have affected the stage of embryonic development at egg collection in the current study is the breeder flock age (61 and 28 weeks at the start of experiment I and II,

respectively). When a breeder flock becomes older, sequence length decreases and consequently, the number of first eggs of a sequence increases (Fasenko et al. 1992a). An embryo in the first egg of a sequence is often further developed than embryos in subsequent eggs (Fasenko et al. 1992a). Another factor that may affect the difference in the average developmental stage and also the variation in the stage of embryonic development at egg collection is environmental temperature (Fasenko et al., 1999). Experiment I was conducted when outside temperature was around 28°C during the day, whereas in experiment II, outside temperature was around 18°C. The outside temperature of 28°C probably increased the temperature in the breeder house above temperatures at which embryos continue development (around 20°C; Edwards, 1902). Fasenko et al. (1999) also found that embryos of eggs that stayed longer (3.5 to 6.5 hours) in the nests at an environmental temperature of 28°C were more developed than eggs collected just after oviposition (stage EG11.67 vs. EG10.38, respectively). Both breeder flock age and environmental temperature may have affected the average stage of embryonic development and variation in the stage of embryonic development at egg collection in the current experiments and, therefore, affected the results of prestorage incubation on hatchability.

Egg weight loss most likely did not affect the results of prestorage incubation in the current study because in experiment I and II, no interaction was found between storage duration and prestorage incubation for total egg weight loss during storage and incubation. Although the results of prestorage incubation were different between the two experiments in the current study, in both experiments, the treatment group with the longest storage duration and prestorage incubation had numerical the highest egg weight loss.

Although in experiment II hatchability of prolonged stored eggs was improved after prestorage incubation, hatchability was still lower than hatchability of short-term stored eggs. It can be hypothesized, therefore, that prestorage incubation is unable to compensate fully for the negative effects of prolonged egg storage. Changes in the egg characteristics, such as the increase in albumen pH (Lapão et al., 1999), the reduction in albumen height (Shenstone, 1968; Burley and Vadehra, 1989), and the reduction in the strength of the vitelline membrane (Fromm, 1966), that occur during storage are not prevented by prestorage incubation. Changes in internal egg quality during storage may have consequences for the protection of the embryo against micro-organisms and for the availability of nutrients for the embryo during incubation and, therefore, may affect hatchability as well as chick quality.

In experiment I, prestorage incubation increased chick quality in terms of chick length independent of storage duration. This difference in chick length between the control and treated group may be caused by a difference in hatch time. In the current study, hatch time was not taken into account and chick quality variables were all measured at the same time. Chicks that hatch first are normally longer than chicks that hatch late (Willemsen et al., 2008). Prestorage incubation increased embryonic development and, therefore, chicks of the prestorage incubation

treatment probably hatched earlier than chicks of the control treatment. This may explain why chick length of the treated group was higher at the moment of measurement than chick length of the control group. In experiment II, prestorage incubation decreased chick quality in terms of the percentage of second-grade chicks independent of storage duration, which cannot be caused by a difference in hatch time. In experiment II, prestorage incubation also negatively affected embryonic mortality from days 18 to 19 of incubation independent of storage duration. The physiology behind these negative effects of prestorage incubation is unknown.

Between the two experiments, there was a numerical difference in chick length and chick body weight. This numerical difference is caused by breeder flock age. At the start of experiment I, breeder flock age was 61 weeks. However, at the start of experiment II, breeder flock age was 28 weeks. Breeder flock age affects egg weight and, therefore, also chick length (Hill, 2001) and chick body weight (Tona et al., 2004; Willemsen et al., 2008).

The current study showed that the effect of prestorage incubation on hatchability depends on storage duration, and that the effect of prestorage incubation on hatchability can be positive or negative. It was also shown that prestorage incubation can be positive or negative for chick quality independent of storage duration. In the current study, the stage of embryonic development was used to investigate what happened with the embryos during prestorage incubation and to investigate whether the stage of embryonic development at egg collection or after prestorage incubation, or both, could explain the results. It seems that prestorage incubation has a positive effect on hatchability after a prolonged egg storage duration when embryos are in a developmental stage below EG10 at egg collection instead of a more advanced stage of development. Factors, such as breeder flock age and environmental temperatures, affect the stage of embryonic development at egg collection (Fasenko et al., 1992a, 1999) and, therefore, affect the results of prestorage incubation. Because different factors affect the stage of embryonic development at egg collection and small changes in prestorage incubation duration have a large effect on the results, prestorage incubation should be used with care.

Acknowledgments

The authors would like to thank the students and colleagues (Adaptation Physiology Group, Wageningen University, Wageningen, the Netherlands and HatchTech Incubation Technology B.V., Veenendaal, the Netherlands) that assisted during the two experiments. They also like to thank Lagerwey hatchery (Lunteren, the Netherlands) and the Van Doren family (Breeder Farm, Zeeland, the Netherlands) for their cooperation.

Chapter 3

Influence of Air Composition during Egg Storage on Egg Characteristics, Embryonic Development, Hatchability, and Chick Quality

I.A.M. Reijrink[1], L.A.G. van Duijvendijk[2], R. Meijerhof[3], B. Kemp[2], and H. van den Brand[2]

[1]HatchTech Incubation Technology B.V., P.O. Box 256, 3900 AG Veenendaal, the Netherlands; [2]Adaptation Physiology Group, Wageningen Institute of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands; [3]Poultry Performance Plus, Kleine Enkweg 1, 7383 DB Voorst, the Netherlands.

Submitted to Poultry Science

Abstract

Egg storage beyond 7 days is associated with an increase in incubation duration and a decrease in hatchability and chick quality. Negative effects of prolonged egg storage may be caused by changes in the embryo, by changes in egg characteristics, or by both. An adjustment in the storage air composition may reduce negative effects of prolonged egg storage, as it may prevent changes in the embryo and in egg characteristics.

An experiment was conducted to investigate the effects of high CO_2concentrations or a low O_2 concentration in the storage air on egg characteristics, embryonic development, hatchability, and chick quality. Eggs were stored for 14 days in 4 different storage air compositions: normal air (control; 20.9% O_2, 0.05% CO_2, 78.1% N_2), 0.74% CO_2 treatment (20.8% O_2, 0.74% CO_2, 77.5% N_2), 1.5% CO_2 treatment (20.6% O_2, 1.5% CO_2, 77.0% N_2), or 3.0% O_2 treatment (3.0% O_2, 0.04% CO_2, 96.0% N_2). The storage temperature was 16°C and the relative humidity was 75%. Results showed that the change in albumen pH and albumen height between ovipsition and the end of storage was less in the 0.74% and 1.5% CO_2 treatments than in the control and 3.0% O_2 treatments ($P < 0.001$ and $P < 0.001$, respectively). None of the treatments affected the stage of embryonic development on day 4 of incubation, hatchability or chick quality on the day of hatch in terms of body weight, yolk-free body mass, and chick length. Although high CO_2 concentrations in the storage air had a positive effect on albumen height and albumen pH, it is concluded that the storage air compositions, studied in the current study, do not affect embryonic development, hatchability, or chick quality, when eggs are stored for 14 days at a storage temperature of 16°C.

Key words: egg storage, carbon dioxide, oxygen, hatchability, chick quality

Introduction

Storage of hatching eggs beyond 7 days is associated with an increase in incubation duration (Mather and Laughlin, 1976; Tona et al., 2003), a decrease in hatchability (Becker, 1964; Merritt, 1964; Fasenko et al., 2001b; Tona et al., 2004; Yassin et al., 2008), and a decrease in chick quality (Byng and Nash, 1962; Merritt, 1964; Tona et al., 2003, 2004). The cause of these negative effects is unclear. During egg storage, embryo viability declines, likely due to an increase in cell death (Arora and Kosin, 1968; Bloom et al., 1998). In addition, egg characteristics change due to a loss of CO_2 and water from the egg. Albumen pH increases from about 7.6 to about 9.0 within 4 days of storage (Lapão et al., 1999). Yolk pH increases from about 6.0 to about 6.5 (Shenstone, 1968), albumen viscosity decreases (Shenstone, 1968; Burley and Vadehra, 1989), and the strength of the yolk membrane decreases during storage as well (Fromm, 1966). These changes in embryo viability and egg characteristics may be related to the negative effects of prolonged egg storage on hatchability and chick quality (Becker et al., 1968; Meijerhof, 1992; Reijrink et al., 2008). The negative effects of prolonged egg storage may be decreased by adjustment of the storage air composition. An increased CO_2 concentration in the storage air prevents an increase in albumen pH and a decrease in albumen height. A lower albumen pH may have a positive effect on embryo viability because the optimal pH for embryonic development is in the range of 7.9 to 8.4 (Gillespie and McHanwell, 1987). In addition, maintenance of albumen height may have a positive effect on the available proteins during embryonic development (Hurnik et al., 1978). A low O_2 concentration in the storage air, which is obtained by an increase in the N_2 concentration, may reduce the metabolic rate of the embryo and, therefore, energy use. When energy use is decreased, the embryo may be able to survive a longer storage duration. In several studies, the effect of storage air composition on hatchability (Becker et al., 1963; Becker, 1964; Proudfoot, 1964 a, b, 1965; Krueger et al., 1965; Warren et al., 1965; Gordon and Siegel, 1966; Becker et al., 1967, 1968; Kosin and Konishi, 1973) and chick quality (Becker, 1964; Proudfoot, 1964a, 1965; Becker et al., 1968) were investigated. In most of these studies, eggs were stored in plastic bags with or without additional N_2 to adjust the storage air composition. These treatments had a positive effect on hatchability when storage duration was beyond 7 days (Becker, 1964; Proudfoot, 1964a, b, 1965; Gordon and Siegel, 1966; Becker et al., 1967, 1968; Kosin and Konishi, 1973). Because the relative humidity and CO_2 concentration both increase when eggs are stored in plastic bags, it is difficult to determine which change or changes in the storage air composition had a positive effect on hatchability.

The current experiment was conducted to investigate the effects of high CO_2 concentrations or a low O_2 concentration in the storage air on egg characteristics, embryonic development, hatchability, and chick quality when eggs were stored for 14 days at a temperature of 16°C and a relative humidity of 75%.

Materials and Methods

This experiment was approved by The Institutional Animal Care and Use Committee of Wageningen University.

Breeder Flock

Eggs from a Ross 308 broiler breeder flock at 38 weeks were used. The male to female ratio was 1:10. Birds were fed restricted diets according to the standard Ross guidelines. The light schedule included 15 hours of light and 9 hours of darkness. The lights were off between 1800 h and 0300 h.

Egg Collection and Storage

The day before egg collection, all eggs were cleared from the nests just before the lights were turned off. Eggs were collected the next day (n=5,890), 5 hours after the lights were turned on. Eggs were randomly assigned to 4 separate climate chambers (Verstegen et al., 1987) and stored for 14 days. The storage air composition differed among the four climate chambers: control treatment (20.9% O_2, 0.05% CO_2, 78.1% N_2; n=1,312), 0.74% CO_2 treatment (20.8% O_2, 0.74% CO_2, 77.5% N_2; n=1,431), 1.5% CO_2 treatment (20.6% O_2, 1.5% CO_2, 77.0% N_2; n=1,555), and 3.0% O_2 treatment (3.0% O_2, 0.04% CO_2, 96.0% N_2; n=1,578).

The control treatment was ventilated with 100 litres of fresh air per minute, the 0.74% CO_2 treatment with 50 litres per minute, the 1.5% CO_2 treatment with 5 litres per minute, and the 3.0% O_2 treatment was not ventilated. The O_2 and CO_2 concentrations in the climate chambers were measured every 9 minutes as described by Lourens et al. (2007). The low O_2 concentration was obtained by injecting N_2 into the climate chambre. The 0.74% and 1.5% CO_2 concentrations were obtained by injecting CO_2 into the climate chambers. CO_2 and N_2 gas were injected in the climate chambers as described by Taylor et al. (1956) with a continuous flow. The flow was adjusted manually when the CO_2 or O_2 concentrations deviated from the desired value. The storage air temperature and relative humidity were measured every 9 minutes and was maintained at 16°C and 75%, respectively.

Egg Weight and Egg Weight Loss during Storage

On the day of oviposition (**d -14**) and on the day of setting (**d 0**), 88 eggs per treatment were weighed individually. Egg weight loss during storage was calculated by the following formula:

(((Egg weight d -14 – Egg weight d 0) / Egg weight d -14) * 100) [1]

Egg Characteristics and Embryonic Development on the Day of Oviposition and during Storage

On the day of oviposition, before the start of the storage treatment (d -14), albumen height, albumen pH, yolk pH, and the stage of embryonic development were determined in 14 randomly selected eggs. The day before the eggs were set (d -1), the same measurements were performed in 20 eggs per treatment. The height of the thick albumen was measured in the middle (Benton and Brake, 1996) with a tripod meter (QCD device, Technical Services and Supplies, York, UK). Albumen was separated from the yolk and both were homogenized with a vortex (YellowLine TTS2, Omnilabo International B.V., Breda, the Netherlands). Thereafter, albumen and yolk pH were measured with a Seven Easy pH meter (Mettler Toledo, Schwerzenbach, Switzerland). The filter ring technique, as described by Gupta and Bakst (1993), was used to isolate the embryo from the yolk membrane. After isolation, the embryo was flushed with buffered saline to remove yolk residue. The dorsal and ventral side of the embryo were examined with a stereo microscope (Olympus SZ61, Olympus Nederland B.V., Zoeterwoude, the Netherlands) to determine the stage of embryonic development according to the classification table of Eyal-Giladi and Kochav (1976) (EG). This classification table measures embryonic development on the basis of morphogenetic movements on the surface of the embryo (Fasenko et al., 1992a).

Incubation

Eggs (n = 5796; 5716 + 80 eggs for analysis on day 4 of incubation) were set into 40 setter trays, which contained at least 144 eggs and maximum 150 eggs (with 3 exceptions: 86, 61, and 37 eggs per setter tray). Each setter tray contained eggs from only one treatment. The eggs were incubated in two setters with a capacity for 4,800 eggs (HT-4,800, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands). Both setters contained eggs of all treatments (2,792 eggs and 3,004 eggs per setter). In each setter, one egg per treatment (4 eggs per setter) was provided with a temperature sensor (NTC Thermistors: type DC 95, Thermometrics, Somerset, UK). Heat-conducting paste (Dow Corning® 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) and a piece of tape were used to attach the sensor to the egg's equator. Average eggshell temperature was maintained at 37.8°C until day 20 of incubation (Lourens et al., 2005). Fertility of the eggs that were provided with a temperature sensor was checked on day 8 of incubation. Relative humidity varied between 45% and 60% and the inlet and outlet valve of the setter were controlled to maintain the CO_2 concentrations below 0.35%. Eggs were turned 90° every hour until day 18 of incubation. On day 18 of incubation, all eggs that contained a living embryo were transferred to hatcher baskets. One setter tray was split in two equal halves and eggs in each half were transferred to one hatcher basket. Per treatment, 30 eggs were individually placed in hatching boxes (10 x 10 cm) to know which chick hatched from which egg. The chicks that hatched in the individual boxes were used to determine chick quality.

The 30 eggs were selected from 2 setter trays out of one setter and hatched in one hatcher. The remaining eggs were hatched in two hatchers (HT-4,800, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands). From day 20 of incubation onward, the air temperature was maintained at 36.7ºC and relative humidity was maintained above 45% in both hatchers. The inlet and outlet valve of the hatchers were controlled to maintain the CO_2 concentrations below 0.35%.

Egg Weight Loss during Incubation

The 88 eggs that were weighed per treatment at d -14 and d 0 were weighed individually again on day 18 of incubation (**d 18**). Egg weight loss during incubation was determined by the following formula:

(((Egg weight d 0 – Egg weight d 18) / Egg weight d 0) * 100) [2]

Total egg weight loss was calculated by the following formula:

(((Egg weight d -14 – Egg weight d 18) / Egg weight d -14) * 100) [3]

Albumen pH and Embryonic Development during Incubation

On day 4 of incubation, albumen pH and the stage of embryonic development were measured in 20 eggs per treatment. Albumen pH was measured as described earlier. The embryo was released from extra embryonic membranes and the dorsal and ventral side of the embryo were examined with a stereo microscope (Olympus SZ61, Olympus Nederland B.V., Zoeterwoude, the Netherlands) to determine the stage of embryonic development according to the classification table of Hamburger and Hamilton (1951) (**HH**). This classification table measures embryonic development on the basis of external factors, such as the number of somite pairs, changes in the wings, legs, visceral arches, feather germs, and eyelids; and the length of the beak and toes.

Embryonic Mortality and Hatchability

On day 8 and 18 of incubation, eggs were candled and after 527 hours of incubation, all un-hatched eggs were collected. Clear eggs, which were removed during candling, and un-hatched eggs were opened to macroscopically determine infertility or the stage of embryonic mortality. The stages of embryonic mortality were determined as described by Reijrink et al. (2009). Fertility was calculated as a percentage of set eggs. Hatchability (first- and second-grade chicks) was calculated as a percentage of set eggs and as a percentage of fertile eggs. Embryonic mortality was calculated as a percentage of fertile eggs.

Incubation Duration and Chick Quality

From day 19.5 of incubation onward, all chicks that emerged from the eggs were recorded every 4 hours to calculate incubation duration. Incubation duration was defined as the interval between the beginning of incubation and the emergence of the chick from the egg. Chicks that hatched in the individual hatching boxes were used to measure chick quality, 12 hours after the chick emerged from the egg. Chick quality was measured in terms of body weight, chick length, yolk-free body mass, residual yolk weight, and navel quality. Chick length was defined as the length from the tip of the beak to the implantation of the nail on the middle toe (Hill, 2001; Willemsen et al., 2008). Navel quality was analyzed and given a score of 1 if the navel was completely closed and clean; a score of 2 if the navel was discolored (color different from skin color) or opened to a maximum of 2 mm, or both; and a score of 3 was given if the navel was discolored or opened more than 2 mm, or both. After body weight, chick length, and navel quality were measured, chicks were euthanized with CO_2 to measure residual yolk weight. Residual yolk weight was subtracted from body weight to calculate yolk-free body mass. Chicks that were not used for chick quality measurements were classified as first- or second-grade chicks based on physical parameters. A chick was classified as a first-grade chick if the chick was clean, dry, free of deformities or lesions, had bright eyes (Tona et al., 2004), and if the chick was given a navel score of 1 or 2. Other chicks were classified as second-grade chicks. The percentage of second-grade chicks was calculated as a percentage of total hatched chicks.

Statistical Analysis

Data were analyzed as a complete randomized design with four treatments. The distribution of the means and residuals were examined to check model assumptions. Egg weight, egg weight loss, body weight, chick length, yolk-free body mass, and residual yolk weight were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with treatment as class variable and egg or chick as the smallest experimental unit. Unfertilized eggs and eggs containing dead embryos were excluded from the data when egg weight and egg weight loss were analyzed. A log transformation was used for egg weight loss during storage, egg weight loss during incubation, and total egg weight loss to obtain normal distributed data. Egg weight at setting was introduced as a covariate when body weight, chick length, yolk-free body mass, and residual yolk weight were analyzed.

To investigate the change in albumen height and yolk pH between the day of oviposition (d -14) and last day of storage (d -1), the average measured value on d -14 was subtracted from the values measured on d -1. The delta's for albumen height and yolk pH were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with treatment as class variable and egg as the smallest experimental unit. To investigate the change in albumen pH between the day of oviposition and last day of storage and between the day of oviposition and day 4 of

incubation, the average measured value on d –14 was subtracted from the values measured on d -1 and d 4. The delta's for albumen pH were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with day of analysis (d -1 or d 4), treatment, and their interaction as class variables and egg as the smallest experimental unit. The stage of embryonic development on the last day of storage and on day 4 of incubation, were analyzed using the Kruskal Wallis nonparametric test (PROC NPAR1WAY, SAS Institute Inc., 2004) with treatment as class variable. Navel quality was analyzed using logistic regression analysis (PROC LOGISTIC, SAS Institute Inc., 2004) with treatment as class variable. Incubation duration, fertility, hatchability, embryonic mortality, and percentage of second-grade chicks were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with treatment, incubator, and their interaction as class variables and setter tray as the smallest experimental unit. An arcsine transformation was used for the percentage of second-grade chicks to obtain normal distributed data. For all continuous variables, least squares means were compared using Bonferroni adjustments for multiple comparisons. Values are expressed as least squares means ± SEM. The main factors and interaction were analyzed for significance at $P \leq 0.05$. The interaction was excluded from the model when $P > 0.05$.

Results

Egg Weight and Egg Weight Loss

Fresh egg weight did not differ among treatments (Table 1). Eggs of the 0.74% CO_2 treatment lost more weight during storage than eggs of the other treatments ($P < 0.001$). Eggs of the control and 3.0% O_2 treatment lost more weight during storage than eggs of the 1.5% CO_2 treatment. Egg weight loss during incubation and total egg weight loss did not differ among treatments.

Table 1. Fresh egg weights and the effect of storage air composition on egg weight loss during storage and incubation

Treatment	Fresh egg weight, g	Egg weight loss during storage[1], %	Egg weight loss during incubation[1], %	Total egg weight loss[1], %
Control	63.92	1.24[b]	10.44	11.55
0.74% CO_2	63.74	1.39[a]	10.52	11.76
1.5% CO_2	63.93	1.11[c]	10.47	11.46
3.0% CO_2	62.97	1.22[b]	10.81	11.90
SEM	0.43	0.02	0.18	0.19
n	306	306	306	306
***P*-value**	0.36	<0.001	0.39	0.30

[a-c]Least squares means within a column lacking a common superscript differ ($P \leq 0.05$). [1]Transformed from log values back to original least squares means.

Egg Characteristics

On day of oviposition, albumen height was 7.13 mm, albumen pH was 8.13, and yolk pH was 6.06. An interaction was found between day of analysis and treatment for the change in albumen pH ($P < 0.001$; Table 2). The change in albumen pH between day of oviposition and last day of storage was different for all treatments. However, the change in albumen pH between day of oviposition and day 4 of incubation was not different among the treatments. On the last day of storage, albumen pH was 9.59 for the 3.0% O_2 treatment, 9.43 for the control treatment, 8.70 for the 0.74% CO_2 treatment, and 8.48 for the 1.5% CO_2 treatment. On day 4 of incubation, albumen pH was 9.07 for the 3.0% O_2 treatment, 9.10 for the control treatment, 9.16 for the 0.74% CO_2 treatment, and 9.10 for the 1.5% CO_2 treatment.

The change in albumen height between day of oviposition and last day of storage was higher for the control and 3.0% O_2 treatments than for the 0.74% and 1.5% CO_2 treatments ($P < 0.001$). The change in yolk pH between day of oviposition and last day of storage did not differ among the treatments.

Table 2. Effect of storage air composition on the change (Δ) in albumen height and yolk pH between the day of oviposition and last day of storage and the effect of day of analysis (d -1 and d 4), storage air composition, and their interaction on the change (Δ) in albumen pH between the day of oviposition and the last day of storage and between the day of oviposition and day 4 of incubation

Treatment	Δ Albumen height, mm	Δ Albumen pH	Δ Yolk pH
Day of analysis, d			
-1		0.92	
4		0.98	
SEM		0.02	
Storage air treatment			
Control	-3.53^{a}	1.13	0.28
0.74% CO_2	-1.57^{b}	0.80	0.26
1.5% CO_2	-1.44^{b}	0.66	0.23
3.0% O_2	-3.52^{a}	1.20	0.29
SEM		0.02	
Day of analysis, d x Storage air treatment			
-1 x Control		1.30^{b}	
-1 x 0.74% CO_2		0.57^{d}	
-1 x 1.5% CO_2		0.35^{e}	
-1 x 3.0% O_2		1.46^{a}	
4 x Control		0.97^{c}	
4 x 0.74% CO_2		1.03^{c}	
4 x 1.5% CO_2		0.97^{c}	
4 x 3.0% O_2		0.94^{c}	
SEM	0.25	0.03	0.02
n	71	149	73
***P*-value**			
Day of analysis		0.009	
Storage air treatment	<0.001	<0.001	0.25
Interaction		<0.001	

$^{a\text{-}e}$Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$).

Embryonic Development

On the day of oviposition, 6.7% of the embryos was at developmental stage EG8, 40.0% of the embryos was at developmental stage EG9, and 53.3% of the embryos was at developmental stage EG10. On the last day of storage and on day 4 of incubation, the stage of embryonic development did not differ among the treatments (Table 3 and 4, respectively).

Table 3. Number and percentage of embryos in each stage of embryonic development for the storage air compositions on the day before setting (d -1)

			Stage of embryonic development[1,2]				
Treatment		**Total**	**EG7**	**EG8**	**EG9**	**EG10**	**EG11**
Control	n	15	0	0	2	12	1
	%	100	0.0	0.0	13.3	80.0	6.7
0.74% CO_2	n	14	0	2	6	6	0
	%	100	0.0	14.2	42.9	42.9	0.0
1.5% CO_2	n	15	1	3	3	7	1
	%	100	6.7	20.0	20.0	46.6	6.7
3.0% O_2	n	14	1	0	6	7	0
	%	100	7.1	0.0	42.9	50.0	0.0

[1]According to Eyal-Giladi and Kochav (EG; 1976). [2]The distribution among the stages of embryonic development were not different among treatments on the day before setting (d -1) ($P = 0.07$).

Table 4. Number and percentage of embryos in each stage of embryonic development for the storage air compositions on day 4 of incubation

			Stage of embryonic development[1,2]			
Treatment		**Total**	**HH19**	**HH20**	**HH21**	**HH22**
Control	n	16	1	3	10	2
	%	100	6.2	18.8	62.5	12.5
0.74% CO_2	n	15	0	6	6	3
	%	100	0.0	40.0	40.0	20.0
1.5% CO_2	n	18	3	5	9	1
	%	100	16.7	27.8	50.0	5.5
3.0% O_2	n	19	0	3	12	4
	%	100	0.0	15.8	63.2	21.0

[1]According to Hamburger and Hamilton (HH; 1951). [2]The distribution among the stages of embryonic development were not different among treatments ($P = 0.17$).

Incubation Duration, Fertility, and Hatchability

There was a small, but significant effect of treatment on incubation duration. Chicks of the 3.0% O_2 treatment hatched 2 hours earlier than chicks of both CO_2 treatments ($P = 0.002$; Table 5). Incubation duration of the control treatment did not differ from other treatments. Fertility, hatchability, and embryonic mortality did not differ among treatments.

Table 5. Effect of storage air composition on incubation duration, fertility, hatchability of set eggs, hatchability of fertile eggs, and embryonic mortality

						Embryonic mortality, % of fertile eggs						
Treatment	**n[1]**	**Incubation duration, h**	**Fertility, %**	**Hatchability of set eggs, %**	**Hatchability of fertile eggs[2], %**	**d 1 to 2**	**d 3**	**d 4 to 9**	**d 10 to 17**	**d 18 to 19**	**d 20**	**d 21**
Control	9 (1,272)	505[ab]	94.1	83.8	89.1	2.5	1.8	3.7	1.0	0.4	0.3	0.2
0.74% CO_2	10 (1,391)	506[a]	94.6	84.9	89.7	1.8	1.6	3.7	0.6	0.7	0.6	0.6
1.5% CO_2	11 (1,515)	506[a]	94.9	85.9	90.6	2.9	1.2	2.2	0.6	0.6	0.5	1.0
3.0% O_2	10 (1,538)	504[b]	93.6	84.7	90.5	2.2	2.0	2.2	0.9	0.5	0.8	0.5
SEM		0.5	0.7	1.1	1.0	0.4	0.3	0.7	0.2	0.2	0.2	0.2
***P*-value**		0.002	0.55	0.57	0.67	0.28	0.30	0.17	0.37	0.86	0.39	0.18

[a-b]Least squares means within a column lacking a common superscript differ ($P \leq 0.05$). [1]n is the number of setter trays with the number of total eggs per treatment within brackets. [2]Differences between hatchability of fertile eggs and total embryonic mortality are caused by candling mistakes and a few cracked eggs.

Chick Quality

Percentage of second-grade chicks, body weight, chick length, and navel quality did not differ among treatments (Table 6). Chicks of the 3.0% O_2 treatment had a higher residual yolk weight than chicks of the control treatment ($P = 0.002$). Residual yolk weight of the 0.74% and 1.5% CO_2 treatment did not differ from the other treatments.

Table 6. Effect of storage air composition on chick quality variables

							Navel quality[4,5]		
Treatment	**Second-grade chicks, arsin**	**Second-grade chicks[1,2], %**	**Body weight[3], g**	**Chick length[3], cm**	**Yolk-free body mass[3], g**	**Residual yolk[3], g**	**1**	**2**	**3**
Control	0.02	0.04	45.3	19.6	40.3	5.09[b]	39.7	50.0	10.3
0.74% CO_2	0.02	0.04	45.5	19.6	40.3	5.31[ab]	54.4	43.9	1.7
1.5% CO_2	0.02	0.04	45.4	19.5	40.1	5.39[ab]	56.1	38.6	5.3
3.0% O_2	0.01	0.01	45.5	19.6	39.9	5.70[a]	56.1	38.6	5.3
SEM	0.01		0.1	0.04	0.2	0.11			
n			229	229	221	221	229		
***P*-value**	0.82		0.61	0.56	0.23	0.002	0.14		

[a-b]Least squares means within a column lacking a common superscript differ ($P \leq 0.05$). [1] Transformed back to original least squares means. [2] Number of second-grade chicks as a percentage of total number of hatched chicks. [3] Corrected in the statistical model for egg weight at setting. [4] Navel quality scored with score 1 to 3. [5] Percentage of chicks with navel score 1, 2, or 3 (total percentage per row is 100%).

Discussion

One of the hypotheses of the current study was that a high CO_2 concentration would improve hatchability and chick quality after prolonged egg storage because an increased CO_2 concentration in the storage air reduces CO_2 loss from the egg and reduces the changes in albumen pH and albumen height. Another hypothesis of the current study was that a low O_2 concentration in the storage air would reduce the embryo's metabolic rate and, therefore, energy use during storage.

In the current study, the change in albumen height and albumen pH were reduced by the increase of the CO_2 concentration in the storage air. The CO_2 concentration of 1.5% maintained albumen pH at 8.48 which is near the pH range from 7.9 to 8.4 measured in the extracellular

space of the embryo during early incubation by Gillespie and McHanwell (1987). However, the 1.5% CO_2 treatment did not improve hatchability or chick quality. This suggests that the increase in albumen pH to a pH around 9.0, which normally occurs within the first 4 days of storage, and the decrease in albumen height are not involved in the negative effects of prolonged egg storage on hatchability and chick quality.

The 3.0% O_2 treatment also did not affect hatchability and chick quality. This shows that embryos can survive low O_2 concentrations during storage. It also suggests that a storage temperature of 16°C most likely reduced the metabolism of the embryo to a level that was low enough to survive a storage duration of 14 days. A decrease in the O_2 concentration had no additional effect.

As in the current study, Kosin and Konishi (1973) did not find an effect of a 1.5% CO_2 concentration in the storage air on hatchability, when storage time was 14 days and a comparable experimental setup as in the current study was used. On the other hand, several authors found a positive effect of storing eggs in plastic bags, in which CO_2 and H_2O gradually increased, on hatchability when storage time was beyond 7 days (Becker, 1964; Gordon and Siegel, 1966; Becker et al., 1967, 1968). In a few other studies, storage of eggs in plastic bags with or without additional N_2 gas only had a pronounced effect on hatchability when the storage duration was beyond 19 days (Proudfoot, 1964a, b, 1965; Kosin and Konishi, 1973). It is, therefore, possible that an albumen pH around 9.0 has a negative effect on embryo viability, but that it was unable to prove this in the current study because storage duration was not long enough. An embryo may be able to maintain an effective barrier between the pH of the inside of the embryo and its exterior (albumen and yolk), but this ability may not be endless because cells may run out of energy when storage duration is too long. Consequently, the embryo may not have enough energy left to survive during incubation (Walsh et al., 1995). How long embryos can be stored without negative consequences may depent on factors that affect embryo viability, such as age of the breeder flock (Mather and Laughlin, 1979), strain (Yoo and Wientjes, 1991), and storage conditions (Arora and Kosin, 1968). In the current study, hatchability was relatively high in comparison to hatchability results of other studies in which egg storage was prolonged (Fasenko et al., 2001; Reijrink et al., 2009). This suggests that the age of the breeder flock and/or strain that was used in the current study positively affected embryo viability and the sensitivity of the embryo to prolonged egg storage.

Although albumen pH was different among the treatments on the last day of storage, albumen pH on day 4 of incubation was comparable for all treatments. It is known that albumen pH increases to a pH around 9.0 during the first 24 hours of incubation (Benton and Brake, 1996). In the current study, albumen pH was only measured on the last day of storage and on day 4 of incubation. Therefore, it is unknown how the pH of the different treatments changed during early incubation. However, it can be assumed that the albumen pH increased to a pH level

around 9.0 during the first 24 hours of incubation in all treatments. The albumen pH during egg storage may not be important for embryo survival during incubation after prolonged egg storage, but the albumen pH during early incubation may be crucial. Maintenance of the albumen pH within the range of 7.9 to 8.4 during early incubation may prevent abnormal development and/or embryonic mortality. In the current study, albumen pH of the different treatments were probably comparable soon after the onset of incubation and this may also be the reason why the treatments did not affect hatchability and chick quality.

In the current study, egg weight loss during storage differed among the treatments. Egg weight loss is influenced by relative humidity, temperature, and the conductivity of the eggshell (Meijerhof, 1994). Relative humidity and temperature were comparable in the different climate chambers. Therefore, conductivity of the eggshell must have been different among the treatments, although eggs were randomized and egg weight loss during incubation did not differ among the treatments. However, it can be assumed that the differences in egg weight loss during storage did not affect hatchability or chick quality because total egg weight loss was not different among the treatments and total egg weight loss of all treatments was close to the optimal egg weight loss of 11.5% (Meir and Ar, 1987; Hulet et al., 1987).

In the current study, the storage air composition did not affect chick quality on the day of hatch in terms of body weight, chick length, and yolk-free body mass. Proudfoot (1964a, 1965), Becker (1964), and Becker et al. (1968), also did not find an effect of storage air composition on chick quality in terms of body weight at hatch and Proudfoot (1965) and Becker et al. (1968) also did not find an effect of storage air composition on subsequent performance.

In conclusion, the storage air compositions, studied in the current study, do not affect embryonic development, hatchability, and chick quality on the day of hatch in terms of body weight, chick length, and yolk-free body mass when eggs are stored for 14 days at a storage temperature of 16°C.

Acknowledgments

We would like to thank Marcel Heetkamp, Ilona van den Anker, and Sonja de Vries (Adaptation Physiology Group, Wageningen University, Wageningen, the Netherlands) for their assistance during the experiment. We also like to thank Lagerwey hatchery (Lunteren, the Netherlands) and the Van Haeren family (Breeder Farm, Wehl, the Netherlands) for their cooperation.

Chapter 4

Influence of Egg Storage Duration and Preincubation Warming Profile on Embryonic Development, Hatchability, and Chick Quality

I.A.M. Reijrink[1], D. Berghmans[2], R. Meijerhof[3], B. Kemp[2], and H. van den Brand[2]

[1]HatchTech Incubation Technology B.V., P.O. Box 256, 3900 AG Veenendaal, the Netherlands; [2]Adaptation Physiology Group, Wageningen Institute of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands; [3]Poultry Performance Plus, Kleine Enkweg 1, 7383 DB Voorst, the Netherlands.

Reprinted by the permission of the Poultry Science Association
Poultry Science (2010) 89:1225-1238

Abstract

When eggs are stored beyond 7 days, hatchability and chick quality decrease. The cause of the negative effects of prolonged egg storage is unclear. The negative effects may be caused by a decrease in embryo viability due to an increase in cell death. The optimal time and curve of preincubation warming (the preincubation warming profile) may be different for eggs stored over short and long periods of time because embryo viability depends on egg storage duration. The aim of the current study was to investigate whether preincubation warming profiles affect embryonic development, hatchability, and chick quality when eggs are stored for a short or prolonged time. Two experiments were conducted. In both experiments, a 2 x 2 completely randomized design was used with: two storage durations (4 and 14 days at 17°C in experiment I and 4 and 13 days at 19°C in experiment II) and 2 preincubation warming profiles (within 4 or 24 hours from storage temperature to 37.8°C).

In experiment I, results suggested that the effect of preincubation warming profile on hatchability depended on storage duration. However, because a low number of eggs were used in this experiment, these differences were not significant. In experiment II, the interaction between storage duration and preincubation warming profile was observed for embryonic mortality during the first 2 days of incubation and hatchability ($P = 0.006$; $P = 0.01$, respectively). When storage duration was 13 days, embryonic mortality during the first 2 days of incubation decreased by 4.4% and hatchability increased by 5.7% when the 24-h preincubation warming profile was used instead of the 4-h preincubation warming profile. However, no effect of preincubation warming profile was observed when storage duration was 4 days. In both experiments, chick quality decreased when storage duration increased but was not affected by preincubation warming profile. In conclusion, a slow preincubation warming profile is beneficial for hatchability when storage duration is prolonged, but does not affect chick quality.

Key words: storage duration, preincubation warming profile, embryonic development, hatchability, chick quality

Introduction

Due to the variable market demand for 1-day-old chicks in the poultry industry and the maximum hatchery capacity, the total length of egg storage varies between a few days and several weeks. Egg storage beyond 7 days is associated with a delay in hatch time (Mather and Laughlin, 1976; Tona et al., 2003), a decline in hatchability (Becker, 1964; Merritt, 1964; Fasenko et al., 2001b; Tona et al., 2004; Yassin et al., 2008), and a decline in chick quality (Tona et al., 2003, 2004). Tona et al. (2003) determined the negative effects of prolonged egg storage on chick quality in terms of physical parameters on the day of hatch such as appearance, activity, and quality of the navel area (Willemsen et al., 2008), and they also measured relative growth during the first week of life. Tona et al. (2004) determined the negative effects of prolonged egg storage on chick quality in terms of weekly body weight until the slaughter age. Parameters such as appearance, activity, and quality of the navel area, are often used in commercial hatcheries to identify second-grade chicks (unsalable chicks) on the day of hatch. Besides body weight and other physical parameters used by Tona et al. (2003), it is also possible to determine the negative effect of prolonged egg storage on chick quality on the day of hatch with a quantitative method, such as yolk-free body mass (Wolanski et al., 2004; Lourens et al., 2005) or chick length (Hill, 2001; Wolanski et al., 2004; Molenaar et al., 2008).

Negative effects of prolonged egg storage on hatchability and chick quality vary with breeder flock age. Yassin et al. (2008) showed that prolonged egg storage (8 to 14 days at the hatchery) decreased hatchability in the eggs of young breeders (25 to 30 weeks) more than in eggs of old breeders (51 to 60 weeks) (0.8 vs. 0.4% per storage day). However, Meijerhof et al. (1994), Elibol et al. (2002), and Tona et al. (2004) showed that the decrease in hatchability was higher for old breeders (59, 52/53, and 45 weeks, respectively) than for young breeders (37, 31/30, and 35 weeks, respectively). These results are difficult to explain because the source for the negative effects of prolonged egg storage is unclear. Negative effects may be caused by changes in the embryo, in the egg characteristics, or by both (Becker et al., 1968; Meijerhof, 1992; Reijrink et al., 2008). To prevent changes in the embryo, eggs are normally stored below temperatures at which morphological development continues. Edwards (1902) reported this temperature is below 20°C, whereas Funk and Biellier (1944) suggested that this temperature is below 27°C.

At the onset of incubation, eggs need to be warmed from the storage temperature to the incubation temperature. The preincubation warming profile is the time and curve used to increase the internal egg temperature from the storage temperature to the incubation temperature. Slow preincubation warming prevents condensation on eggs at the onset of incubation, but the effects of the preincubation warming profile on embryo viability are unknown. Some authors have suggested that it is beneficial to warm eggs quickly to the incubation temperature (Wilson, 1991; Renema et al., 2006) because a prolonged time at temperatures below 35°C may increase embryonic mortality and/or abnormal embryonic development (Wilson, 1991; Renema et al.,

2006). On the other hand, Hodgetts (1999) suggested that eggs should be warmed slowly to reduce a temperature shock to the embryos. The optimal preincubation warming profile may be different for eggs stored for short and long periods of time. Although eggs are stored at a temperature below 20°C, embryo viability declines during egg storage likely due to cell death (Arora and Kosin, 1968; Bloom et al., 1998). The increase in cell death over time may cause that long-term stored embryos have less viable embryonic cells left at the onset of incubation than short-term stored embryos. Embryos that contain less viable embryonic cells at the onset of incubation may, therefore, be more sensitive to nonoptimal preincubation warming profiles. The aim of the current study was to investigate whether preincubation warming profiles affect embryonic development, hatchability, and chick quality when eggs are stored for a short or prolonged time.

Materials and Methods

Experimental Design

Two experiments were conducted. Experiment I was a randomized complete block design, with 3 comparable batches as blocks. Each block had a 2 x 2 completely randomized design that included: 2 storage durations (4 and 14 days) and 2 preincubation warming profiles (within 4 or 24 hours from 17°C to 37.8°C). Experiment II was a 2 x 2 completely randomized design that included: 2 storage durations (4 and 13 days) and 2 preincubation warming profiles (within 4 or 24 hours from 19°C to 37.8°C). The Institutional Animal Care and Use Committee of Wageningen University approved experiment I. Experiment II was conducted under practical circumstances in a commercial hatchery.

Breeder Flock

For the 2 experiments, 2 different Ross 308 broiler breeder flocks were used. Birds were fed restricted diets according to standard Ross guidelines. In experiment I, the age of the breeder flock varied from 41 weeks in batch 1 to 50 weeks in batch 3. The male to female ratio was 1:11. The light schedule included 16 hours of light and 8 hours of darkness. The lights were off between 1800 h and 0400 h. In experiment II, the age of the breeder flock varied between 28 to 29 weeks. The male to female ratio was 1:8. The light schedule included 15 hours of light and 9 hours of darkness. The lights were off between 1600 h and 0100 h.

Egg Collection and Egg Storage

The day before egg collection, all eggs were cleared from the nests before darkness. In experiment I, eggs were collected between 0400 h and 0800 h, 4 or 14 days prior to setting. Eggs (n = 865) were stored on paper trays at a temperature between 16°C and 18°C. In experiment II, eggs were collected between 0830 h and 1000 h, 4 or 13 days prior to setting. Eggs (n = 8,400) were stored on setter trays at a temperature between 18°C and 20°C. In both experiments, the relative humidity in the storage room was not measured and not controlled. In experiment I, egg weight and the stage of embryonic development were measured on the day of oviposition and on the last day of storage.

Egg Weight and Egg Weight Loss

In experiment I, all eggs were weighed individually on the day of oviposition and on the last day of storage. Egg weight loss that occurred between the day of oviposition and the last day of storage was expressed as a percentage of fresh egg weight.

Embryonic Development

In experiment I, the stage of embryonic development was determined in 10 to 15 eggs per batch and storage duration on the day of oviposition and on the last day of storage. The filter ring technique, as described by Gupta and Bakst (1993), was used to isolate the embryo from the yolk membrane. After isolation, the embryo was flushed with buffered saline to remove yolk residue. The dorsal and ventral side of the embryo were examined with a stereomicroscope (Olympus SZ61, Olympus Nederland B.V., Zoeterwoude, the Netherlands) to determine the stage of embryonic development according to the classification table of Eyal-Giladi and Kochav (1976) (EG). This classification table measures embryonic development on the basis of morphogenetic movements on the surface of the embryo (Fasenko et al., 1992a).

Preincubation Warming Profiles

At the onset of incubation, 2 preincubation warming profiles were used to warm eggs from the storage temperature to an internal egg temperature of 37.8°C:

1. Preincubation warming from storage temperature to 37.8°C was conducted in a setter (HT-4,800, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands) in about 4 hours. Air velocity was maintained at 0.6 m/s and eggs were turned 90° every 10 minutes. This treatment is called the **4-h preincubation warming profile.**
2. Preincubation warming from the storage temperature to 37.8°C was conducted in a setter (HT-4,800, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands) in about 24 hours. Within the first 12 hours, the internal egg temperature increased from storage temperature to 28°C. Within the second 12 hours, the internal egg temperature increased

from 28°C to 37.8°C. Air velocity was maintained at 0.3 m/s and eggs were turned 90° every 10 minutes. This treatment is called the **24-h preincubation warming profile.**

During preincubation warming, the internal egg temperature was measured in 2 eggs per storage duration and preincubation warming profile. A temperature sensor (NTC Thermistors, type DC 95, Thermometrics, Somerset, UK) was inserted in the egg for 15 mm into the egg through a hole of 3 mm in diameter in the eggshell in the middle of the blunt end of the egg. Eggs, that were used to measure the internal egg temperature were excluded from the experiment at the end of preincubation warming.

To start the incubation process of all treatments at the same time, preincubation warming of the 24-h preincubation warming profile began about 20 hours earlier than preincubation warming of the 4-h preincubation warming profile. Preincubation warming ended and incubation began when the internal egg temperature of all treatments reached 37.8°C (after about 24 and 4 hours).

Incubation

Eggs of both preincubation warming profiles were incubated seperately. Two setters (HT-4,800, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands) were used and contained eggs from both 4 and 14 or 13 days of storage. In experiment I, about 75 eggs were distributed over one setter tray, which can hold 150 eggs. Between each egg, an empty space was included. Per batch, 3 setter trays were used per setter and eggs from 4 and 14 days of storage were mixed. Because eggs of different storage durations were mixed, it was impossible to use the setter tray as smallest experimental unit. Thus, the egg was the smallest experimental unit in experiment I. In experiment II, 56 setter trays filled with 150 eggs each were used. Each setter tray contained eggs of 1 storage duration. Therefore, the setter tray could be used as the smallest experimental unit in experiment II. In both experiments, the eggshell temperature of 2 eggs per storage duration was measured (4 eggs per setter) with a temperature sensor (NTC Thermistors, type DC 95, Thermometrics, Somerset, UK) in each setter. Heat-conducting paste (Dow Corning® 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) and a piece of tape were used to attach the sensor to the egg's equator. Eggshell temperature was maintained at 38.1°C from days 0 to 8 of incubation, at 37.9°C from days 8 to 12 of incubation, and at 37.6°C from days 12 to 18 of incubation, to reach an average internal egg temperature of 37.8°C. Extra eggs were used to determine whether the internal egg temperature was 37.8°C. These extra eggs were excluded from the experiment. Relative humidity varied between 50% and 60% and the inlet and outlet valve of the setter were controlled to maintain CO_2 concentrations below 0.35%. Eggs were turned 90° every hour until day 18 of incubation.

In experiment I, all eggs that contained a living embryo were individually placed in hatching boxes (10 x 10 cm), which were placed in 1 hatcher (HT-4,800, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands) on day 18 of incubation. In experiment II, eggs of 1 setter tray that contained a living embryo were transferred to hatcher baskets. A setter tray was split in 2 equal halves and eggs in each half were transferred to 1 hatcher basket. Two hatchers were used (HT-4,800, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands) and the eggs of 1 setter tray were placed in the same hatcher. In both experiments, the hatcher temperature was set at 36.7°C. Relative humidity varied between 50% and 55% and the inlet and outlet valve of the hatcher were controlled to maintain CO_2 concentrations below 0.35%. In experiment I, egg weight loss, extra embryonic membrane development, embryonic development, embryonic mortality, hatchability, incubation duration, and chick quality were measured and in experiment II embryonic mortality, hatchability, and chick quality were measured.

Egg Weight Loss

In experiment I, all eggs were weighed individually on day 18 of incubation. Egg weight loss that occured between the last day of storage and day 18 of incubation was expressed as a percentage of the egg weight on the last day of storage. Total egg weight loss was the sum of egg weight losses during storage and incubation.

Extra Embryonic Membrane Development and Embryonic Development

In experiment I, extra embryonic membrane development and embryonic development were measured on day 7 of incubation. Per batch, 13 eggs per storage duration and preincubation warming profile were taken from the setter to measure the wet weight of the allantois, amnion, and embryo and to measure embryo length and the stage of embryonic development. Eggs were carefully opened at the sharp end of the egg and the content of the eggshell was emptied into a Petri dish. Yolk membrane was dissected from the allantois, amnion, and embryo. Next, the total weight of the allantois, amnion, and embryo was measured. The allantois was dissected from the amnion and embryo and the total weight of the amnion and embryo was measured. The amnion was dissected from the embryo and the embryo weight was measured. The embryo weight was subtracted from the total weight of the amnion and embryo to calculate the amnion weight. Total weight of amnion and embryo was subtracted from the total weight of the allantois, amnion, and embryo to calculate the allantois weight.

The length of the embryo was defined as the length from the tip of the beak to the tip of the middle toe. The dorsal and ventral side of the embryo were examined with a stereomicroscope (Olympus SZ61, Olympus Nederland B.V., Zoeterwoude, the Netherlands) to determine the stage of embryonic development according to the classification table of Hamburger and

Hamilton (1951) (**HH**). This classification table measures embryonic development on the basis of external factors, such as the number of somite pairs, changes in the wings, legs, visceral arches, feather germs, and eyelids; and the length of the beak and toes.

Embryonic Mortality and Hatchability

In both experiments, eggs were candled on day 8 and day 18 of incubation. After 522 hours of incubation, all un-hatched eggs were collected. Clear eggs, which were removed during candling, and unhatched eggs were opened to macroscopically determine infertility or the stage of embryonic mortality. Because fertility was determined macroscopically, it is possible that an embryo that died during storage was classified as an infertile egg. The stages of embryonic mortality were determined as described by Reijrink et al. (2009). Fertility was calculated as a percentage of set eggs. Hatchability (first- and second-grade chicks) was calculated as a percentage of set eggs and as a percentage of fertile eggs. Embryonic mortality was calculated as a percentage of fertile eggs. In experiment I, embryonic mortality was divided into 3 categories: embryonic mortality from days 0 to 9, from days 10 to 17, and from days 18 to 21 of incubation.

Incubation Duration and Chick Quality

In experiment I, hatch time and chick quality of each individual chick were measured. From day 19.5 of incubation and onward, chicks that emerged from the eggs were recorded individually every 2 hours to calculate incubation duration. Incubation duration is defined as the interval between the end of preincubation warming and the emergence of the chick from the egg. For each treatment, the time required to hatch the first 50% of all hatched chicks was calculated from the time the first chick hatched until the time when more than 50% of all chicks hatched. The time required to hatch the last 50% of all hatched chicks was calculated from the time more than 50% of all chicks hatched until the time the last chick hatched.

Twelve hours after each individual chick emerged from the egg, chick quality was measured in terms of rectal temperature, body weight, chick length, navel quality, residual yolk weight, heart weight, liver weight, stomach weight, and intestine weight. Chick length was defined as the length from the tip of the beak to the implantation of the nail on the middle toe (Hill, 2001; Willemsen et al., 2008). Rectal temperature was measured with a digital thermometer (VT 1831, Microlife Veterinary Thermometer, Microlife, Widnau, Switzerland) inserted 1 cm into the cloaca. Navel quality was analyzed and given a score of 1 if the navel was completely closed and clean; a score of 2 if the navel was discolored (color different from skin color) or opened to a maximum of 2 mm, or both; and a score of 3 was given if the navel was discolored or opened more than 2 mm, or both. After rectal temperature, body weight, chick length, and

navel quality were measured, chicks were euthanized with CO_2 to measure the residual yolk and organ weights. Residual yolk weight was subtracted from body weight to calculate yolk-free body mass.

In experiment II, all hatched chicks were classified as first- or second-grade chicks based on physical parameters. A chick was classified as a first-grade chick if the chick was clean, dry, free of deformities or lesions, had bright eyes (Tona et al., 2004), and if the chick was given a navel score of 1 or 2. Other chicks were classified as second-grade chicks. The percentage of second-grade chicks was calculated as a percentage of total chicks hatched.

Statistical Analysis

Distributions of the means and residuals were examined to check model assumptions. In experiment I, the duration of the 2 preincubation warming profiles were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with the preincubation warming profile as the class variable. Percentage of egg weight loss during storage and egg weight loss per storage day were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with batch, storage duration, and their interaction as class variables. A log transformation was conducted for egg weight loss during storage, and egg weight loss per storage day to obtain normal distributed data. The stage of embryonic development on the day of oviposition and the last day of storage were analyzed using logistic regression analysis (PROC LOGISTIC, SAS Institute Inc., 2004) with day of analysis, storage duration, and their interaction as class variables. The rest of the data from experiment I were analyzed as a 3 x 2 x 2 randomized complete block design with 3 batches (block), 2 storage durations, and 2 preincubation warming profiles. Egg or chick was the smallest experimental unit. Egg weight, egg weight loss during incubation, total egg weight loss, allantois weight, amnion weight, embryo weight, embryo length, incubation duration, body weight, chick length, yolk-free body mass, rectal temperature, and organ weights were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with batch, storage duration, preincubation warming profile, and their interactions as class variables. A log transformation was conducted for egg weight loss during incubation and total egg weight loss to obtain normal distributed data. Fresh egg weight was introduced as a covariate when embryo weight and embryo length on day 7 of incubation were analyzed. Embryo weight on day 7 of incubation was introduced as a covariate when allantois weight and amnion weight were analyzed. Fresh egg weight was introduced as a covariate when body weight, chick length, and yolk-free body mass were analyzed. Yolk-free body mass was introduced as a covariate when organ weights were analyzed. Values are expressed as least squares means ± SEM. Least squares means were compared using the Bonferroni adjustment for multiple comparisons. All main factors and interactions were analyzed for significance at the $P \leq 0.05$ level. Except for the storage duration and preincubation warming profile interaction, all interactions were excluded

stepwise from the model when $P > 0.05$.

In experiment I, a general linear regression could not be used to analyze hatchability data because the egg was the smallest experimental unit. Fertility, hatchability, embryonic mortality, stage of embryonic development on day 7 of incubation, and navel quality were analyzed using logistic regression analysis (PROC LOGISTIC, SAS Institute Inc., 2004) with batch, storage duration, preincubation warming profile, and their interactions as class variables. All the main factors and interactions were analyzed for significance at the $P \leq 0.05$ level. Except for the storage duration and preincubation warming profile interaction, all interactions were excluded stepwise from the model when $P > 0.05$.

Data from experiment II were analyzed as a 2 x 2 completely randomized design with 2 storage durations and 2 preincubation warming profiles. A setter tray containing 150 eggs, was the smallest experimental unit. Fertility, hatchability, embryonic mortality, and the percentage of second-grade chicks were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with storage duration, preincubation warming profile, and their interaction as class variables. An arcsine transformation was conducted for embryonic mortality from days 10 to 17 of incubation to obtain normal distributed data. Main factors and interaction were analyzed for significance at the $P \leq 0.05$ level. Least squares means were compared using the Bonferroni adjustment for multiple comparisons. Values are expressed as least squares means ± SEM.

Results

Embryonic Development on Day of Oviposition and Last Day of Storage

In experiment I, no interaction was found between day of analysis and storage duration (Table 1). Day of analysis and storage duration did not affect the stage of embryonic development ($P = 0.28$ and $P = 0.51$, respectively).

Table 1. Number and percentage of embryos at each stage of development for the interaction between day of analysis and storage duration (experiment I)

				Stages of embryonic development[1,2]				
Day of analysis	**Storage duration, d**		**Total**	**EG9**	**EG10**	**EG11**	**EG12**	**EG13**
Day of oviposition	4	n	24	2	12	9	1	0
		%	100	8.3	50.0	37.5	4.2	0.0
Day of oviposition	14	n	31	3	14	14	0	0
		%	100	9.6	45.2	45.2	0.0	0.0
Last day of storage	4	n	47	2	19	22	4	0
		%	100	4.3	40.4	46.8	8.5	0.0
Last day of storage	14	n	42	4	18	19	0	1
		%	100	9.5	42.9	45.2	0.0	2.4

[1]According to Eyal-Giladi and Kochav (1976). [2]The distribution among stages of embryonic development were not different for the interaction between day of analysis and storage duration ($P = 0.47$).

Preincubation Warming Profiles

In experiment I, preincubation warming duration of the 4-h preincubation warming profile was 4 hours and 22 minutes (± 43 minutes, SD). Preincubation warming duration of the 24-h preincubation warming profile was 23 hours and 53 minutes (± 38 minutes, SD). The preincubation warming profiles differed ($P < 0.001$; Figure 1). In experiment II, preincubation warming duration of the 4-h preincubation warming profile was 4 hours and 8 minutes and preincubation warming duration of the 24-h preincubation warming profile was 23 hours and 26 minutes.

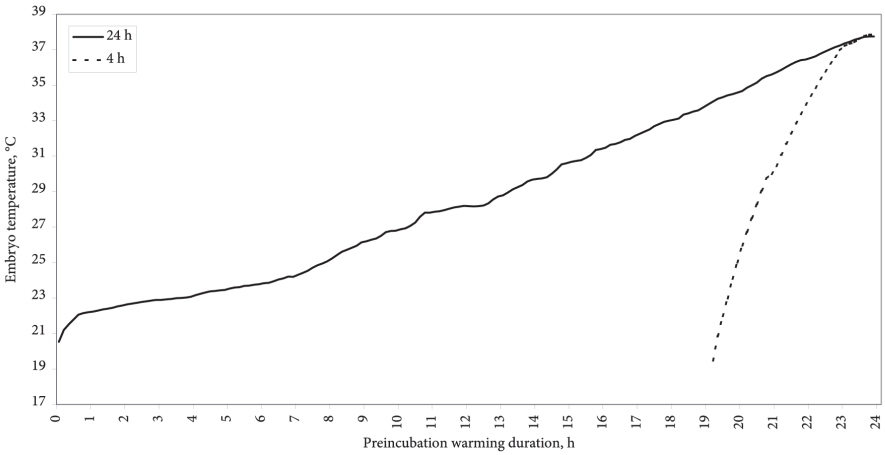

Figure 1. The 4- and 24-h preincubation warming profiles.

Egg Weight and Egg Weight Loss

In experiment I, no interaction between storage duration and preincubation warming profile was observed for fresh egg weight, egg weight loss during incubation, and total egg weight loss (Table 2). Fresh egg weight did not differ between the two storage days. Eggs stored for 4 days lost 0.53% less weight during storage than eggs stored for 14 days ($P < 0.001$). Eggs stored for 4 days lost 0.01% more weight per storage day than eggs stored for 14 days ($P < 0.001$). Storage duration did not affect egg weight loss during incubation. Total egg weight loss was 0.42% lower for eggs stored for 4 days than for eggs stored for 14 days ($P = 0.03$). Fresh egg weight did not differ between the two preincubation warming profiles. Egg weight loss during incubation and total egg weight loss were lower for the 4-h preincubation warming profile than for the 24-h preincubation warming profile ($P < 0.001$ and $P < 0.001$, respectively).

Table 2. Fresh egg weights and the effect of storage duration and preincubation warming profile on egg weight losses during storage and incubation (experiment I)

Treatment	Fresh egg weight, g	Egg weight loss during storage, log	Egg weight loss during storage[1], %	Egg weight loss per storage day, log	Egg weight loss per storage day[1], %	Egg weight loss during incubation, log	Egg weight loss during incubation[1], %	Total egg weight loss, log	Total egg weight loss[1], %
Storage duration, d									
4	65.0	-1.21[b]	0.30	-2.60[a]	0.07	2.32	10.17	2.34[b]	10.38
14	64.7	-0.19[a]	0.83	-2.83[b]	0.06	2.31	10.07	2.38[a]	10.80
SEM	0.2	0.01		0.01		0.01		0.01	
Preincubation warming profile, h									
4	65.1					2.28[b]	9.78	2.33[b]	10.28
24	64.6					2.34[a]	10.38	2.39[a]	10.91
SEM	0.2					0.01		0.01	
Storage duration, d x preincubation warming profile, h									
4 x 4	65.4					2.28	9.78	2.30	9.97
4 x 24	64.6					2.36	10.59	2.39	10.91
14 x 4	64.8					2.28	9.78	2.36	10.59
14 x 24	64.6					2.33	10.28	2.40	11.02
SEM	0.3					0.02		0.02	
n	548	548		548		378		378	
***P*-value**									
Storage duration	0.24	<0.001		<0.001		0.54		0.03	
Preincubation warming profile	0.06					<0.001		<0.001	
Interaction	0.25					0.29		0.26	

[a-b]Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$). [1]Transformed back to original least squares means.

Extra Embryonic Membrane and Embryonic Development

In experiment I, no interaction between storage duration and preincubation warming profile was observed for allantois weight and amnion weight (Table 3). Storage duration did not affect allantois weight and amnion weight. Amnion weight was 0.18 g higher for the 4-h preincubation warming profile than for the 24-h preincubation warming profile ($P = 0.05$). The preincubation warming profile did not affect allantois weight.

An interaction between storage duration and preincubation warming profile was observed for embryo weight ($P = 0.05$) and embryo length ($P = 0.03$). Eggs stored for 4 days and warmed with a 24-h preincubation warming profile had a higher embryo weight than embryos of the other treatments. Eggs stored for 4 days and warmed with the 4-h preincubation warming profile had comparable embryo weights as eggs stored for 14 days and warmed with the 24-h preincubation warming profile. Embryos of these 2 treatments had a higher weight than embryos of the 14-days storage duration and 4-h preincubation warming profile. These differences among treatments were also observed in embryo length, except that embryos of the 14-days storage duration and 24-h preincubation warming profile were as long as embryos of the 4-days storage duration and 24-h preincubation warming profile.

No interaction between storage duration and preincubation warming profile was observed for the stage of embryonic development. Embryos stored for 4 days were more developed than embryos stored for 14 days ($P < 0.001$). Embryos of the 24-h preincubation warming profile were more developed than embryos of the 4-h preincubation warming profile ($P < 0.001$).

Table 3. Effect of storage duration and preincubation warming profile on extra embryonic membrane development and embryonic development on day 7 of incubation (experiment I)

					Stages of embryonic development[3,4,5]			
Treatment	**Allantois[1], g**	**Amnion[1], g**	**Embryo weight[2], g**	**Embryo length[2], mm**	**HH28**	**HH29**	**HH30**	**HH31**
Storage duration, d								
4	2.57	1.51	1.08	31.0	0.0	18.4	29.0	52.6
14	2.47	1.56	0.96	29.6	14.5	40.6	31.9	13.0
SEM	0.17	0.05	0.01	0.2				
Preincubation warming profile, h								
4	2.56	1.63^{a}	0.95	29.5	11.1	41.7	33.3	13.9
24	2.49	1.45^{b}	1.10	31.1	2.8	16.4	27.4	53.4
SEM	0.18	0.06	0.01	0.2				
Storage duration, d x Preincubation warming profile, h								
4 x 4	2.70	1.61	1.02^{b}	30.4^{b}	0.0	29.0	44.7	26.3
4 x 24	2.44	1.42	1.14^{a}	31.5^{a}	0.0	7.9	13.1	79.0
14 x 4	2.42	1.65	0.87^{c}	28.6^{c}	23.5	55.9	20.6	0.0
14 x 24	2.53	1.48	1.05^{b}	30.7^{ab}	5.7	25.7	42.9	25.7
SEM	0.25	0.08	0.02	0.2				
n	85	96	123	145	144			
***P*-value**								
Storage duration	0.72	0.55	<0.001	<0.001	<0.001			
Preincubation warming profile	0.80	0.05	<0.001	<0.001	<0.001			
Interaction	0.39	0.96	0.05	0.03	0.97			

$^{a\text{-}c}$Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$). [1]Corrected in the statistical model for embryo weight on day 7 of incubation. [2]Corrected in the statistical model for fresh egg weight. [3]According to Hamburger and Hamilton (HH; 1951). [4]Percentage of embryos at each stage of development (total percentage per row is 100%). [5]The distributions among developmental stages differed between storage duration ($P \leq 0.05$) and between preincubation warming profiles ($P \leq 0.05$).

Incubation Duration

No interaction between storage duration and preincubation warming profile was observed for incubation duration. Within the same preincubation warming profile, chicks from the 4-days storage duration hatched earlier than chicks from the 14-days storage duration (Figure 2). Chicks from the 4-days storage duration hatched after 486.3 hours of incubation and chicks from the 14-days storage duration hatched after 493.8 hours of incubation ($P < 0.001$). Chicks from the 24-h preincubation warming profile hatched after 486.7 hours of incubation and chicks from the 4-h preincubation warming profile hatched after 493.4 hours of incubation ($P < 0.001$).

The time required to hatch the first 50% of all hatched chicks was 16 hours for the 4-days storage duration and 4-h preincubation warming profile, 4-days storage duration and 24-h preincubation warming profile, and 14-days storage duration and 4-h preincubation warming profile. The time required to hatch the first 50% of all hatched chicks was 18 hours for the 14-days storage duration and 24-h preincubation warming profile. The time required to hatch the last 50% of all hatched chicks was 22 hours for the 4-days storage duration and 4-h preincubation warming profile, 24 hours for 14-days storage duration and 4-h preincubation warming profile, and 14-days storage duration and 24-h preincubation warming profile, and 32 hours for 4-days storage duration and 24-h preincubation warming profile. The last mentioned treatment hatched the last 50% of all hatched chicks within 16 hours, except for the last chick, which needed another 16 hours to hatch.

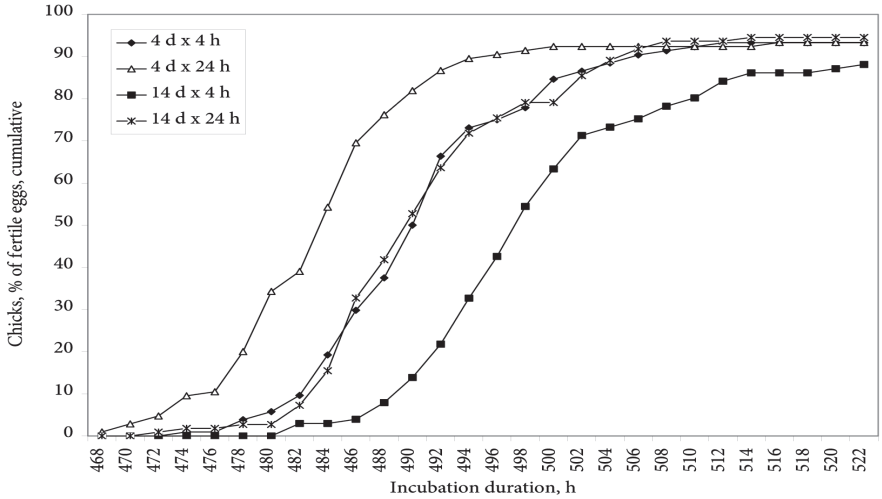

Figure 2. Incubation duration for hatched chicks as a percentage of fertile eggs for the interaction between storage duration and preincubation warming profile.

Fertility and Hatchability

In experiment I, no interaction between storage duration and preincubation warming profile was observed for fertility, hatchability of set eggs, hatchability of fertile eggs, and embryonic mortality during incubation (Table 4). Neither storage duration nor preincubation warming profile affected fertility, hatchability of set eggs, hatchability of fertile eggs, and embryonic mortality during incubation.

Table 4. Effect of storage duration and preincubation warming profile on fertility, hatchability of set eggs, hatchability of fertile eggs, and embryonic mortality (experiment I)

					Embryonic mortality, % of fertile eggs		
Treatment	**n**	**Fertility, %**	**Hatchability of set eggs, %**	**Hatchability of fertile eggs, %**	**d 0 to 9**	**d 10 to 17[1]**	**d 18 to 19**
Storage duration, d							
4	235	88.9	83.0	93.3	5.3	0.0	1.4
14	234	90.2	82.5	91.5	7.6	0.0	1.0
Preincubation warming profile, h							
4	230	89.1	80.9	90.7	7.8	0.0	1.5
24	239	90.0	84.5	94.0	5.1	0.0	0.9
Storage duration, d x preincubation warming profile, h							
4 x 4	117	88.9	82.9	93.3	5.8	0.0	1.0
4 x 24	118	89.0	83.1	93.3	4.8	0.0	1.9
14 x 4	113	89.4	78.8	88.1	9.9	0.0	2.0
14 x 24	121	90.9	86.0	94.6	5.5	0.0	0.0
***P*-value**							
Storage duration		0.88	0.44	0.21	0.28		0.56
Preincubation warming profile		0.99	0.99	0.99	0.75		0.57
Interaction		0.79	0.32	0.27	0.59		0.95

[1]Embryonic mortality from days 10 to 17 of incubation was zero for all treatments and, therefore, no statistics were computed.

In experiment II, an interaction between storage duration and preincubation warming profile was observed for the hatchability of fertile eggs ($P = 0.01$) and embryonic mortality during the first 2 days of incubation ($P = 0.006$; Table 5). The hatchability of fertile eggs was 5.7% higher for the 24-h preincubation warming profile than for the 4-h preincubation warming profile when storage duration was 13 days, whereas the preincubation warming profile did not affect the hatchability of fertile eggs when storage duration was 4 days. Embryonic mortality during the first 2 days of incubation was 4.4% lower for the 24-h preincubation warming profile than for the 4-h preincubation warming profile when storage duration was 13 days, whereas the preincubation warming profile did not affect embryonic mortality during the first 2 days of incubation when storage duration was 4 days. When storage duration increased, fertility decreased by 2.4% ($P < 0.001$), the hatchability of set eggs decreased by 18.1% ($P < 0.001$), embryonic mortality on day 3 of incubation increased by 1.9% ($P < 0.001$), embryonic mortality from days 4 to 9 of incubation increased by 1.5% ($P < 0.001$), embryonic mortality from days 10 to 17 of incubation increased by 0.4% ($P = 0.01$), and embryonic mortality from days 18 to 19 of incubation increased by 0.8% ($P < 0.001$). Embryonic mortality on day 21 of incubation decreased by 0.2% ($P = 0.04$). The hatchability of set eggs was 2.2% higher ($P = 0.04$), and embryonic mortality on day 20 of incubation was 0.7% lower for the 24-h preincubation warming profile than for the 4-h preincubation warming profile ($P < 0.001$).

Chick Quality

In experiment I, no interaction between storage duration and preincubation warming profile was observed for body weight, chick length, yolk-free body mass, navel quality, rectal temperature, and organ weights (Table 6). Chicks of the 4-days storage duration were 0.1 cm longer ($P < 0.001$) and had 0.5 more g of yolk-free body mass ($P = 0.004$) than chicks of the 14-days storage duration. Storage duration did not affect other chick quality variables. The rectal temperature of chicks of the 24-h preincubation warming profile was 0.1°C lower than the rectal temperature of chicks of the 4-h preincubation warming profile ($P = 0.02$). The preincubation warming profile did not affect other chick quality variables.

In experiment II, no interaction between storage duration and preincubation warming profile was observed for the percentage of second-grade chicks (Table 5). The percentage of second-grade chicks was 0.6% higher when storage duration was 13 days than when storage duration was 4 days ($P = 0.02$). The preincubation warming profile did not affect the percentage of second-grade chicks.

Table 5. Effect of storage duration and preincubation warming profile on fertility, hatchability of set eggs, hatchability of fertile eggs, embryonic mortality, and second-grade chicks (experiment II)

					Embryonic mortality, % of fertile eggs								
Treatment	**n[1]**	**Fertility, %**	**Hatchability of set eggs, %**	**Hatchability of fertile eggs[2], %**	**d 1 to 2**	**d 3**	**d 4 to 9**	**d 10 to 17, arsin**	**d 10 to 17[3]**	**d 18 to 19**	**d 20**	**d 21**	**Second-grade chicks[4], %**
Storage duration, d													
4	29	95.3^{a}	88.7^{a}	93.1	2.5	1.6^{b}	1.1^{b}	0.03^{b}	0.1	0.3^{b}	0.7	0.3^{a}	0.7^{b}
13	27	92.9^{b}	70.6^{b}	76.0	14.9	3.5^{a}	2.6^{a}	0.07^{a}	0.5	1.1^{a}	1.0	0.1^{b}	1.3^{a}
SEM		0.4	0.7	0.7	0.5	0.3	0.2	0.01		0.1	0.1	0.1	0.2
Preincubationwarming profile, h													
4	28	94.6	78.6^{b}	82.9	9.8	2.4	1.9	0.05	0.3	0.9	1.2^{a}	0.2	0.9
24	28	93.6	80.8^{a}	86.2	7.6	2.7	1.7	0.05	0.3	0.6	0.5^{b}	0.2	1.0
SEM		0.4	0.7	0.7	0.5	0.3	0.2	0.01		0.1	0.1	0.1	0.2
Storage duration, d x preincubation warming profile, h													
4 x 4	15	95.6	88.6	92.7^{a}	2.6^{c}	1.7	1.0	0.03	0.1	0.4	1.0	0.3	0.6
4 x 24	14	95.0	88.9	93.5^{a}	2.4^{c}	1.6	1.2	0.04	0.2	0.3	0.4	0.2	0.8
13 x 4	13	93.6	68.5	73.2^{c}	17.1^{a}	3.2	2.9	0.08	0.6	1.3	1.4	0.1	1.3
13 x 24	14	92.1	72.6	78.9^{b}	12.7^{b}	3.8	2.3	0.07	0.5	0.8	0.6	0.1	1.2
SEM		0.6	1.0	1.0	0.7	0.4	0.3	0.01		0.1	0.1	0.1	0.3
***P*-value**													
Storage duration		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.01		<0.001	0.09	0.04	0.02
Preincubation warming profile		0.11	0.04	0.002	0.004	0.57	0.56	0.82		0.11	<0.001	0.86	0.88
Interaction		0.42	0.06	0.01	0.006	0.45	0.26	0.40		0.34	0.44	0.53	0.49

$^{a\text{-}c}$Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$). [1]n = number of setter trays that contained 150 eggs at the start of the experiment. [2]Differences between hatchability of fertile eggs and total embryonic mortality are due to candling mistakes and cracked eggs. [3]Transformed back to original least squares means. [4]Number of second-grade chicks as a percentage of total number of hatched chicks.

Table 6. Effect of storage duration and preincubation warming profile on body weight, chick length, yolk-free body mass, rectal temperature, navel quality, and organ weights (experiment I)

				Navel quality[2,3]							
Treatment	**Body weight[1], g**	**Chick length[1], cm**	**Yolk-free bodymass[1], g**	**1**	**2**	**3**	**Rectal temperature, °C**	**Heart[4], g**	**Liver[4], g**	**Stomach[4], g**	**Intestines[4], g**
Storage duration, d											
4	46.5	19.7[a]	40.7[a]	51.1	47.9	1.0	39.6	0.39	1.17	2.49	2.02
14	46.3	19.6[b]	40.2[b]	50.5	43.8	5.7	39.7	0.39	1.16	2.52	2.02
SEM	0.1	0.03	0.1				0.03	0.004	0.01	0.02	0.02
Preincubation warming profile, h											
4	46.4	19.6	40.4	47.3	48.9	3.8	39.7[a]	0.39	1.17	2.50	2.02
24	46.4	19.6	40.4	54.0	43.0	3.0	39.6[b]	0.39	1.16	2.51	2.02
SEM	0.1	0.03	0.1				0.03	0.004	0.01	0.02	0.02
Storage duration, d x Preincubation warming profile, h											
4 x 4	46.5	19.7	40.6	49.0	51.0	0.0	39.7	0.38	1.19	2.47	2.02
4 x 24	46.5	19.7	40.7	53.1	44.9	2.0	39.6	0.39	1.15	2.51	2.02
14 x 4	46.4	19.5	40.2	45.4	46.6	8.0	39.7	0.39	1.16	2.54	2.03
14 x 24	46.3	19.6	40.2	54.8	41.3	3.9	39.7	0.39	1.16	2.51	2.02
SEM	0.1	0.04	0.2				0.04	0.01	0.01	0.03	0.03
n	385	386	254	386			386	253	249	254	253
***P*-value**											
Storage duration	0.12	<0.001	0.004	0.33			0.07	0.71	0.43	0.17	0.86
Preincubation warming profile	0.94	0.64	0.87	0.70			0.02	0.99	0.20	0.67	0.87
Interaction	0.60	0.07	0.94	0.39			0.48	0.58	0.06	0.21	0.95

[a-b]Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$). [1]Corrected in the statistical model for fresh egg weight. [2]Navel quality scored with score 1 to 3. [3]Percentage of chicks with navel score 1, 2, or 3 (total percentage per row is 100%). [4] Corrected in the statistical model for yolk-free body mass.

Discussion

In experiment I of the current study, prolonged egg storage increased incubation duration. The preincubation warming profile did not affect the difference in incubation durations between eggs stored for short or long periods of time. Within the same preincubation warming profile, chicks of the 4-days storage duration hatched earlier than chicks of the 14-days storage duration. Embryonic development was not accelerated or changed by the 24-h preincubation warming profile; embryos had just more time to develop before incubation at 37.8°C began. The internal egg temperature of the 24-h preincubation warming profile was above 27°C for 10 hours before the preincubation warming of the 4-h preincubation warming profile began. When the internal egg temperature is above 27°C, embryos continue morphological development (Funk and Biellier, 1944). Consequently, embryos of the 24-h preincubation warming profile were more advanced than embryos of the 4-h preincubation warming profile throughout incubation, which resulted in a shorter incubation duration when preincubation warming was excluded. Because embryonic development of the 24-h preincubation warming profile was advanced throughout incubation, it is surprising that the amnion weight of this preincubation warming profile was lower than the 4-h preincubation warming profile on day 7 of incubation. The reason for this is unknown, but may be related to the higher egg weight loss during incubation of the 24-h preincubation warming profile. Egg weight loss during incubation, which included egg weight loss during preincubation warming, was higher for the 24-h preincubation warming profile than for the 4-h preincubation warming profile. This phenomenon appears to be a result of time and temperature. At the beginning of the 24-h preincubation warming profile, eggs of the 4-h preincubation warming profile were in the storage room at 18°C for 20 hours, whereas eggs of the 24-h preincubation warming profile were exposed to a linear increase in temperature, which increased egg weight loss (Walsh et al., 1995).

In experiment II, the 24-h preincubation warming profile had a beneficial effect on embryonic mortality during the first 2 days of incubation and on hatchability when storage duration was prolonged. However, the preincubation warming profile did not affect embryonic mortality during the first 2 days of incubation and hatchability when storage duration was short. In experiment I, this interaction between storage duration and preincubation warming profile was not significant. Numerical differences in experiment I suggest that an interaction between storage duration and preincubation warming profile for embryonic mortality during the first 9 days of incubation and hatchability seems reasonable. Regarding hatchability, an interaction between storage duration and preincubation warming profile was also observed by Proudfoot (1966). Half of the eggs were prewarmed at 21-24°C for 18 hours prior to setting and the other half of the eggs were directly set from the storage room, which operated at 10°C to 12°C. Warming eggs prior to setting increased hatchability when eggs were stored for more than 14 days. However, warming eggs prior to setting did not affect hatchability when eggs were stored

for 14 days. The fact that a slow preincubation warming profile had no effect on hatchability when storage duration was short, is in agreement with findings of Van Schalkwyk et al. (1999).

The interaction between storage duration and preincubation warming profile for embryonic mortality during the first 2 days of incubation found in experiment II of the current study suggests that embryos stored for a prolonged time are more sensitive to a nonoptimal preincubation warming profile than embryos stored for a short time. During storage, cell death increases (Arora and Kosin, 1968; Bloom et al., 1998) and this may cause a difference in embryo viability between eggs stored for short or long periods of time. It can be hypothesized that a particular stage of differentiation needs a minimum number of viable embryonic cells to continue embryonic development and growth successfully; otherwise, embryos will develop abnormally or will die within the first days of incubation. Funk and Biellier (1944) suggested that embryos continue morphological development at temperatures above 27°C. Although no morphological development may be recognizable at a temperature below 27°C, Arora and Kosin (1968) showed that mitotic activity in the embryo increased when the temperature increased from 7.2°C to 18.3°C. Within the first 10 hours of the 24-h preincubation warming profile, the internal egg temperature increased from 17°C to 27°C. At these temperatures, embryos may not develop morphologically (Funk and Biellier, 1944) but mitotic activity in the embryo may increase due to a rise in temperature (Arora and Kosin, 1968). We hypothesize that due to an increase in the mitotic activity during the first 10 hours of the 24-h preincubation warming profile, the embryo is able to compensate for cell death that occurred during storage, which improves embryo viability. Embryos of the 4-h preincubation warming profile had little opportunity to compensate for cell death because the temperature was above 27°C within 1 hour of preincubation warming. The inability of the embryo to compensate for cell death before morphological development continued, may have increased embryonic mortality during the first 2 days of incubation in experiment II.

The difference in the age of the breeder flock between experiment I and II may have affected hatchability results, and the decrease in hatchability caused by prolonged egg storage. In experiment I, the age of the breeder flock increased from 41 to 50 weeks and in experiment II, the age of the breeder flock increased from 28 to 29 weeks. Yassin et al. (2008) showed that the average hatchability of set eggs increased to 86% between the ages of 31 to 36 weeks and decreased thereafter. In addition, it was shown that the hatchability of young breeders (25 to 30 weeks) was affected more by prolonged egg storage than the hatchability of old breeders (51 to 60 weeks). In the current study, the hatchability of young breeders (experiment II) was also more affected by prolonged egg storage than the hatchability of old breeders (experiment I). Because the eggs in experiment II were from a young breeder flock, it may be expected that the embryos were less developed at oviposition (Fasenko et al., 1992a) than in experiment I. Less-developed embryos may be more sensitive to prolonged egg storage than more developed

embryos (Fasenko et al., 2001b; Reijrink et al., 2009). The expected difference in the sensitivity of the embryos to prolonged egg storage between experiment I and II may have affected the interaction between storage duration and preincubation warming profile in experiment II and the absence of this interaction in experiment I. However, when storage duration was prolonged, differences in embryonic mortality during the first 9 days of incubation were comparable (4.4%) between the 4-h and 24-h preincubation warming profile in both experiments. Therefore, it seems more likely that the number of eggs used in experiment I was too low to find a significant interaction.

Due to a longer storage duration, egg weight loss during storage was higher for eggs stored for 14 days than for eggs stored for 4 days. A change in the relative humidity during storage may have affected daily egg weight loss as well. However, whether or not the relative humidity changed during storage is unknown because the relative humidity in the storage room was not measured and not controlled. Egg weight loss during storage had a small effect on total egg weight loss; therefore, it can be assumed that the effect of egg weight loss during storage on hatchability or chick quality was negligible (Meijerhof, 1994).

In experiment II, fertility decreased when storage duration increased. Fertility was determined macroscopically and some embryos that died during storage may have been classified as infertile eggs. Thus, embryonic mortality during the first two days of incubation may be underestimated.

Tona et al. (2003, 2004) has shown that prolonged egg storage had a negative effect on chick quality in terms of physical parameters on the day of hatch, relative growth during the first 7 days of life, and weekly body weight until the slaughter age. The negative effect of prolonged egg storage on chick quality was also observed in both experiments in the current study. In experiment I, yolk-free body mass and chick length decreased when storage duration increased. In experiment II, the percentage of second-grade chicks increased when storage duration increased. Results from both experiments showed that yolk-free body mass, chick length, and the percentage of second-grade chicks are meaningful indicators of chick quality. Although a slow preincubation warming profile had a beneficial effect on hatchability when storage duration was prolonged, it had no beneficial effect on chick quality. The preincubation warming profile had a minor effect on rectal temperatures, but did not affect chick quality in terms of yolk-free body mass and chick length.

It can be concluded from the current study that the preincubation warming profile does not affect hatchability when eggs are stored for a short time. However, a slow preincubation warming profile has a beneficial effect on hatchability when storage duration is prolonged due to a reduction in embryonic mortality during the first two days of incubation. Although the preincubation warming profile affects embryonic mortality during the first two days of incubation of stored eggs, chick quality is not affected.

Acknowledgments

We would like to thank Ilona van den Anker, Annelies van der Linden, Woutrien de Bruin, Linda Baarslag (Adaptation Physiology Group, Wageningen University, Wageningen, the Netherlands), Cor Termaat, Albert Goorts, Roos Molenaar, and Conny Maatjens (HatchTech Incubation Technology B.V., Veenendaal, the Netherlands) for their assistance during the experiments. We would also like to thank the Lagerwey hatchery (Lunteren, the Netherlands), Michel Banning (Breeder Farm, Didam, the Netherlands), and Peter van de Beek (Breeder Farm, Vlierden, the Netherlands) for their cooperation. We are also grateful to Mike Hulet (Department of Poultry Science, Penn State University, State College, USA), who reviewed this manuscript and gave useful comments.

Chapter 5

Influence of Egg Warming during Storage and Hypercapnic Incubation on Egg Characteristics, Embryonic Development, Hatchability, and Chick Quality

I.A.M. Reijrink[1], R. Meijerhof[2], B. Kemp[3], and H. van den Brand[3]

[1]HatchTech Incubation Technology B.V., P.O. Box 256, 3900 AG Veenendaal, the Netherlands; [2]Poultry Performance Plus, Kleine Enkweg 1, 7383 DB Voorst, the Netherlands; [3]Adaptation Physiology Group, Wageningen Institute of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands.

Submitted to Poultry Science

Abstract

Negative effects of prolonged egg storage on hatchability and chick quality may be caused by changes in the embryo, in the egg characteristics, or by both. The aim of this experiment was to investigate whether prestorage incubation (PSI), frequent warming during storage (FW), or hypercapnic incubation during the first 5 days of incubation (HI) affect egg characteristics, embryonic development, hatchability, and chick quality. The experiment had a 2 x 2 x 2 randomized design: PSI (yes-no), FW (yes-no), and HI (yes-no). All eggs were stored for 15 days at 16°C and 75% relative humidity. On the second day after oviposition, half of the eggs were incubated for 7 hours (PSI). During storage, half of the eggs were warmed 6 times for 30 minutes in water at 37.8˚C (FW). During the first 5 days of incubation, the CO_2 concentration in the incubator was maintained between 0.70% and 0.80% (hypercapnic incubation), or increased from 0.05% to 0.20% (control).

PSI and FW increased the stage of embryonic development and the number of viable embryonic cells, but these treatments did not have a pronounced effect on egg characteristics, hatchability, or chick quality. HI decreased total albumen pH, which was measured at 18, 42, 66, and 90 hours of incubation, and decreased the percentage of eggs classified as infertile (Δ=1.2%). In contrast, HI retarded embryonic development, decreased hatchability of fertile eggs by 1.3%, but did not affect chick quality.

It can be concluded that both PSI and FW did not improve hatchability and chick quality, although the stage of embryonic development and the number of viable embryonic cells increased due to the treatments. HI decreased total albumen pH, which may be related to the increased number of embryos that continued their development at the onset of incubation. Because HI retarded further embryonic development and decreased hatchability, HI at concentrations between 0.70% to 0.80% during the first 5 days of incubation was probably too long.

Key words: egg storage, warming during storage, hypercapnic incubation, embryonic development, albumen pH

Introduction

After hatching eggs are collected from laying nests, eggs are stored at the breeder farm, transported to and stored at the hatchery, and finally set in an incubator. The storage duration depends on the supply of hatching eggs, hatchery capacity, and the demand for day-old chicks. Normally, commercial hatcheries will set their eggs after 3 to 5 days of storage, but in some situations a hatchery needs to increase the storage duration beyond 7 days. A storage duration beyond 7 days increases incubation duration (Mather and Laughlin, 1976; Tona et al., 2003) and has a negative effect on hatchability (Becker, 1964; Fasenko et al., 2001b; Tona et al., 2004; Yassin et al., 2008) and chick quality (Byng and Nash, 1962; Merritt, 1964; Tona et al., 2003, 2004). The negative effects of prolonged egg storage may be caused by changes in the embryo, in the egg characteristics, or by both (Becker et al., 1968; Meijerhof, 1992; Reijrink et al., 2008).

Fasenko et al. (2001b) hypothesized that embryos at the developmental stage EG13 (Eyal Giladi and Kochav, 1976 (**EG**)) are less sensitive to prolonged egg storage than embryos that are less or further advanced because the developmental stage EG13 is a quiescent stage. In addition, Reijrink et al. (2009) showed that embryos at a developmental stage below EG10 were sensitive to prolonged egg storage. The ability of an embryo to survive prolonged egg storage may depend on the cell activity at a particular stage of development, but may also depend on the number of viable embryonic cells. When the number of viable embryonic cells is low at the onset of incubation due to cell death during storage, particular steps in the embryo's development may be impeded. This may result in abnormal development or embryonic death.

The first aim of the current study was to investigate whether prestorage incubation (**PSI**) and frequent warming during storage (**FW**) affected egg characteristics, embryonic development, hatchability, and chick quality when eggs were stored for 15 days. PSI and FW treatments were both used to see whether the effect was different when eggs were incubated once for a few hours or several times for half an hour.

An embryo that increased development during storage due to PSI or FW may be better able to form an effective barrier between the inside of the embryo (pH ranges from 7.9 to 8.4; Gillespie and McHanwell, 1987) and its exterior (albumen pH around 9.0 and yolk pH around 6.5) during early incubation than a less developed embryo. It is also possible that a more developed embryo decreases the pH in its micro-environment close to the range from 7.9 to 8.4 due to its own CO_2 production. If pH is important for successful early embryonic development, a decrease in albumen pH during early incubation, due to hypercapnic incubation, may also have a positive effect on embryonic development and hatchability.

Sadler et al. (1954) showed that a high CO_2 concentration in the incubator during the first 48 and 72 hours of incubation decreased albumen pH and stimulated embryonic development. Bruggeman et al. (2007) and De Smit et al. (2006, 2008) observed that a gradual increase of CO_2 during the first 10 days of incubation to a concentration of 1.0% or 1.5% accelerated embryonic

development. De Smit et al. (2006, 2008) also observed a positive effect on hatchability. Sadler et al. (1954), Bruggeman et al. (2007), and De Smit et al. (2006, 2008) investigated the effect of hypercapnic incubation on embryonic development and hatchability by using short-term stored eggs, but the beneficial effect of hypercapnic conditions during early incubation may increase when embryo viability is reduced due to prolonged egg storage.

The second aim of the current study was to investigate whether hypercapnic incubation during the first 5 days of incubation (**HI**) affected egg characteristics, embryonic development, hatchability, and chick quality when eggs were stored for 15 days and whether effects of HI depend on the stage of embryonic development at the onset of incubation.

Materials and Methods

Experimental Design

The experiment had a 2 x 2 x 2 randomized design: PSI (yes-no), FW (yes-no), and HI (yes-no). This experiment was approved by the Institutional Animal Care and Use Committee of Wageningen University.

Breeder Flock

Eggs from a Ross 308 broiler breeder flock at 36 weeks were used. The male to female ratio was 1:8.5. Birds were fed restricted diets according to the standard Ross guidelines. The light schedule included 16 hours of light and 8 hours of darkness. Lights were off between 1800 h and 0200 h.

Egg Collection and Storage

The day before egg collection, all eggs were cleared from the nests before the lights were turned off. Eggs were collected at 0700 h and 1300 h until 10,800 eggs were collected. Eggs were stored for 1 night at the breeder farm at a temperature of 18°C. The next day, eggs were transported to the research facility and stored on setter trays in two climate chambers (Verstegen et al., 1987) at a temperature of 16°C and a relative humidity of 75%. Storage duration was 15 days.

Prestorage Incubation

On the second day after oviposition, half of the eggs were incubated for 7 hours. Two incubators (HT-1,408, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands) were used twice (4 batches). In both incubators, 4 eggs were used to measure internal egg temperature. A sensor (NTC Thermistors: type DC 95, Thermometrics, Somerset, UK) was inserted in the egg for 15 mm through a hole of 3 mm in diameter in the eggshell in the middle

of the blunt end of the egg. Eggs were warmed to an internal egg temperature of 37.8°C within 2 hours and this internal egg temperature was maintained for 3 hours. Thereafter, eggs were cooled down in the incubator for 2 hours to an internal egg temperature of 25°C. During incubation, relative humidity varied between 45% and 55% and the CO_2 concentration was 0.04%. After the incubation period, eggs were again stored in climate chambers. Eggs exposed to PSI in 4 batches were mixed before they were assigned to the FW treatment.

Frequent Warming during Storage

During storage, half of the control eggs and half of the eggs that were exposed to PSI were warmed in a water bath, which contained water at 37.8°C. Eggs were warmed every other day between the second and thirteenth day of storage. Before eggs were warmed in water, they were packed in plastic bags (freezing bags, 20 x 30 cm, C1000, Wageningen, the Netherlands) to avoid water contact. The plastic bags were not closed to keep eggs in contact with air during warming. Eggs were warmed for 30 minutes and placed back in the climate chambers after the plastic bags were removed.

Measurements During Storage

Egg Weight and Egg Weight Loss

Egg weight loss was measured on setter tray level. At the start of the experiment, each setter tray contained 150 eggs. The weight of the full setter trays was measured on the day after oviposition (tfd-14) and on the last day of egg storage (tfd-1). To calculate egg weight loss, weight of the empty setter tray (te) was also measured.

Weight loss was calculated with the following formula:

$$(((tf_{d\text{-}14} - te) - (tf_{d\text{-}1} - te)) / (tf_{d\text{-}14} - te)) * 100 \quad [1]$$

Average fresh egg weight was calculated per setter tray with the following formula:

$$(tf_{d\text{-}14} - te) / (\text{the number of eggs per setter tray}) \quad [2]$$

Egg Characteristics

The day after oviposition, 40 eggs were used to measure albumen height, albumen pH, and yolk pH. On day 14 and 15 of storage, 20 eggs per treatment were used for the same measurements. The height of the thick albumen was measured in the middle (Benton and Brake, 1996) with a tripod meter (QCD device, Technical Services and Supplies, York, UK). The albumen was separated from the yolk and both were homogenized with a vortex (YellowLine TTS2, Omnilabo International B.V., Breda, the Netherlands). Thereafter, albumen and yolk pH were measured with a Seven Easy pH meter (Mettler Toledo, Schwerzenbach, Switzerland).

Embryonic Development

In eggs that were used to measure the egg characteristics also the stage of embryonic development was determined. The filter ring technique, as described by Gupta and Bakst (1993), was used to isolate the embryo from the yolk membrane. After isolation, the embryo was flushed with buffered saline to remove yolk residue. The dorsal and ventral side of the embryo were examined with a stereo microscope (Olympus SZ61, Olympus Nederland B.V., Zoeterwoude, the Netherlands) to determine the stage of embryonic development according to the classification table of Eyal-Giladi and Kochav (1976) or Hamburger and Hamilton (1951) (HH). The classification table of Eyal-Giladi and Kochav (1976) measures embryonic development on the basis of morphogenetic movements on the surface of the embryo (Fasenko et al., 1992a). The classification table of Hamburger and Hamilton (1951) measures embryonic development on the basis of external factors, such as the number of somite pairs, changes in the wings, legs, visceral arches, feather germs, and eyelids; and the length of the beak and toes.

Number of Embryonic Cells and Apoptosis Analysis

The isolated embryos stayed in a Petri dish filled with saline (8 g NaCl, 0.2 g KCl, 1.4 g Na_2HPO_4-$2H_2O$, and 0.2 g KH_2PO_4 in 1l Milli-Q water adjusted to a pH of 7.2) for 2 to 2.5 hours. Afterward, 3 embryos were pooled in a 2 ml micro centrifuge tube and 0.5 ml of 0.25% trypsin (0.5% trypsin and 0.53mM EDTA (Cat. No. 15400-054, Invitrogen, http://products.invitrogen.com) was added. The trypsin plus embryos were pipetted up and down for five times with a pasteur pipet and, thereafter, the cell suspension was incubated at 37°C for 10 minutes. During incubation, the cell suspension was pipetted up and down for five times after 3 and 6 minutes. At the end of incubation, the cell suspension was pipetted up and down with a pasteur pipet for 30 seconds and with a Rainin pipet lite of 0.2 ml (Mettler Toledo B.V., Tiel, the Netherlands) for another 60 seconds. After pipetting, 1.5 ml of DMEM (Bio Whittaker DMEM, Cat. No. BE12-604F/U1, Lonza Benelux B.V., Breda, the Netherlands) plus 15% fetal calf serum was added to the cell suspension to neutralize the trypsin. Then, the cell suspension was centrifuged (Eppendorf centrifuge 5415, Merck, Amsterdam, the Netherlands) at a speed of 1500 r/min for 15 seconds. The supernatant was removed and the cell suspension was centrifuged again with 1 ml of saline. The supernatant was removed again and 150 µl of Annexin V binding buffer (1x) (Annexin V-FITC Apoptosis Detection Kit I, Cat. No. 556547, BD Pharmingen^{tm}, http://www.bdeurope.com) was added to the cells. This cell suspension was divided in two parts: 100 µl and 50 µl. The 100 µl was used to measure the percentage of viable embryonic cells, early apoptotic cells, and late apoptotic/necrotic cells. The 50 µl was used to count the total number of nuclei (embryonic cells). To count the total number of nuclei, 5 µl of Triton X-100 (0.5%) (Triton X-100, Cat. No. 76051752, Boom B.V., Meppel, the Netherlands) and 20 µl of Bisbenzimide

(0.2mg/ml diluted in saline) (Bisbenzimide H 33258, Cat. No. 382061, Calbiochem Corporation, http://eshop.emdchemicals.com) was added to the cell suspension. The cell suspension was homogenized and three samples of 10 µl were used to count the total number of nuclei. To count the nuclei a double Bürker Türk counting chamber (W. Schreck, Hofheim/TS, Germany) and a inverted microscope (IM 35, Zeiss, Oberkochen, Germany) with the filter combination BP 135, FT 395 and, LP 397 were used. The total number of nuclei per embryo was calculated with the following formula:

Total number of nuclei per embryo = ((total volume of cell suspension / (number of squares counted[1] * volume of one square[2])) * the average number of nuclei of the three counts) / number of embryos[3]. [3]

[1]Number of squares counted = 72

[2]Volume of one square = 0,004 µl.

[3]Number of embryos = 3.

To measure the percentage of viable embryonic cells, early apoptotic cells, and late apoptotic/necrotic cells, the Annexin V-FITC Apoptosis Detection Kit I (Cat. No. 556547, BD Pharmingentm, http://www.bdeurope.com) was used according to the manufacturer's instructions. The loss of membrane integrity is one of the early steps of the apoptotic cascade and leads to the translocation of a phosphatidylserine (PS) from the cytoplasmic side of the cell to the outer membrane. The Annexin V-FITC Apoptosis Detection Kit I contains FITC Annexin V, which has a high affinity for PS and binds to cells with exposed PS. Propidium Iodide is included in the Annexin V-FITC Apoptosis Detection Kit I to identify the latest stages of apoptosis.

Per sample, 10,000 cells were analyzed by FACScan flow cytometry (XL.MCLtm Flow Cytometer, Beckman Coulter, Woerden, the Netherlands) with excitation and emission wavelength settings at 488 and 530 nm, respectively. Analysis was performed by using EXPO 32 ADC software (Beckman Coulter, Woerden, the Netherlands). To calculate the total number of viable embryonic cells, the total number of embryonic cells was multiplied by the percentage of viable embryonic cells.

Hypercapnic Incubation

After 15 days of storage, eggs were transported to a commercial hatchery (Lagerwey B.V., Lunteren, the Netherlands). For the first 5 days of incubation, eggs were incubated in 4 setters (HT-4,800, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands). All setters contained eggs of all four storage treatments (control, PSI, FW, and PSI plus FW treatment). In 2 of the 4 setters, eggs were incubated under hypercapnic conditions. CO_2 was injected (CO_2

Supply System, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands) into the setter to maintain the CO_2 concentrations between 0.70% and 0.80%. CO_2 was automatically injected in the incubator when the CO_2 concentration decreased below 0.70%. This CO_2 concentration was used to decrease albumen pH, but to stay below the critical concentration of 1.0% (Sadler et al., 1954; Taylor et al., 1956). In the other two setters, CO_2 concentration increased gradually, due to CO_2 production of the embryos, from 0.05% to 0.20% within 5 days of incubation. In all setters, relative humidity was maintained between 65% and 75% for the first 5 days of incubation. Per setter, one egg of each storage treatment (4 in total) was provided with an eggshell sensor. Heat-conducting paste (Dow Corning® 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) and a piece of tape were used to attach the sensor to the egg's equator. During the first 5 days of incubation, average eggshell temperature was maintained at 37.8°C (Lourens et al., 2005). On day 6 of incubation, all eggs were transferred to one setter (HT-57,600, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands). Eggs of all eight treatments were equally divided over four setter trolleys. Per setter trolley, four eggs were provived with an eggshell sensor. In total, two eggs per treatment were provived with an eggshell sensor and average eggshell temperature was maintained at 37.8°C (Lourens et al., 2005) until day 19 of incubation. Relative humidity varied between 35% and 50% and inlet and outlet valve of the setter were controlled to maintain CO_2 concentrations below 0.35%. Eggs were turned 90° every hour from set until day 19 of incubation.

On day 19 of incubation, all eggs that contained a living embryo were transferred to hatcher baskets. A setter tray was split in two equal halves and eggs of each half were transferred to one hatcher basket. All hatcher baskets were divided over 4 hatcher trolleys and placed in one hatcher (HT-28,800, HatchTech Incubation Technology B.V., Veenendaal, the Netherlands). Per hatcher trolley, four eggs were provided with an eggshell sensor and average eggshell temperature was maintained at 37.8°C (Lourens et al., 2005) until the end of day 19 of incubation. Afterward air temperature was maintained at 36.1°C. Relative humidity varied between 50% and 60% and inlet and outlet valve of the hatcher were controlled to maintain the CO_2 concentrations below 0.35%.

Measurements during Incubation

Egg Weight Loss during Incubation

On day 19 of incubation, the full setter trays (tf_{d19}) were weighed again and egg weight loss during incubation was calculated with the following formula:

$$(((tf_{d-1} - te) - (tf_{d19} - te)) / (tf_{d-1} - te)) * 100 \quad [4]$$

Total egg weight loss was the sum of egg weight loss during storage and incubation.

Egg Characteristics and Embryonic Development during Incubation

At 18, 42, 66, and 90 hours of incubation, 20 eggs per treatment were used to determine albumen pH and the stage of embryonic development. A hole was made in the sharp end of the egg to remove the albumen. Albumen pH was measured as described earlier. The embryo was released from the extra embryonic membranes and the dorsal and ventral side of the embryo were examined with a stereo microscope (Olympus SZ61, Olympus Nederland B.V., Zoeterwoude, the Netherlands) to determine the stage of embryonic development according to the classification table of Hamburger and Hamilton (1951).

Embryonic Mortality and Hatchability

On day 6 of incubation, eggs were candled and after 520 hours of incubation, all unhatched eggs were collected. Clear eggs, which were removed during candling, and unhatched eggs were opened to macroscopically determine infertility or stage of embryonic mortality. Stages of embryonic mortality described by Reijrink et al. (2009) were used to classify dead embryos. The number of malpositioned and malformationed embryos were also counted. A malpositioned embryo was defined as an embryo that was further developed than day 17 of incubation, but did not have its head under the right wing. A malformationed embryo had one or more of the following malformations: two limbs, exposed brain, cross beak, or double heads. Malpositioned and malformationed embryos were calculated as a percentage of fertile eggs. The malpositioned and malformationed embryos were already classified as described by Reijrink et al. (2009) and, therefore, the malpositioned and malformationed embryos are double counts. Fertility was calculated as a percentage of set eggs. Hatchability (first- and second-grade chicks) was calculated as a percentage of set eggs or as a percentage of fertile eggs. Embryonic mortality was calculated as a percentage of fertile eggs.

Incubation Duration and Chick Quality

From day 19.5 of incubation and onward, all chicks that were emerged from the eggs were counted per two hatcher baskets (one setter tray) every 4 hours to calculate incubation duration. Incubation duration was defined as the interval between the beginning of incubation and the emergence of the chick from the egg. Per setter tray, the 5^{th}, 30^{th}, 50^{th}, 80^{th}, and 105^{th} chick that emerged from the egg was set apart in the same hatcher to measure chick quality 12 hours after the chick emerged from the egg. Chick quality was measured in terms of body weight, chick length, navel quality, and residual yolk, heart, liver, and stomach weight. Chick length was defined as the length from the tip of the beak to the implantation of the nail on the middle toe (Hill, 2001; Willemsen et al., 2008). Navel quality was analyzed and given a score of 1 if the navel was completely closed and clean; a score of 2 if the navel was discolored (color different from skin color) or opened to a maximum of 2 mm, or both; and a score of 3 was given if the

navel was discolored or opened more than 2 mm, or both. After body weight, chick length, and navel quality were measured, chicks were euthanized with CO_2 to measure residual yolk and organ weights. Residual yolk weight was subtracted from body weight to calculate yolk-free body mass.

All hatched chicks were classified as first- or second-grade chicks based on physical parameters. A chick was classified as a first-grade chick if the chick was clean, dry, free of deformities or lesions, had bright eyes (Tona et al., 2004), and if the chick was given a navel score of 1 or 2. The other chicks were classified as second-grade chicks. The percentage of second-grade chicks was calculated as a percentage of total hatched chicks.

Statistical Analysis

Albumen height, albumen pH, yolk pH, total number of embryonic cells, total number of viable embryonic cells, percentage of early apoptotic cells, and percentage of late apoptotic/necrotic cells did not differ between day 14 and 15 of storage and, therefore, data of both days were pooled for further analysis.

Percentage of egg weight loss during storage, albumen height, albumen pH, and yolk pH at the end of storage were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with PSI, FW, and their interaction as class variables. For the analysis of egg weight loss, setter tray was the smallest experimental unit. For the analysis of egg characteristics, egg was the smallest experimental unit. To investigate differences in the data collected on the first day after oviposition and the last two days of storage for total number of embryonic cells, total number of viable embryonic cells, percentage of early apoptotic cells, and percentage of late apoptotic/necrotic cells, a new class variable was introduced named "group". This was necessary because on the first day after oviposition there was no treatment effect yet. Group was the interaction between day of analysis and treatment. The first day after oviposition was indicated as group 1, whereas at the end of storage the 4 treatments were indicated as group 2 until 5 (Table 2). The total number of embryonic cells and number of viable embryonic cells were ranked from 1 to 47 and from 1 to 46, respectively and rank numbers were analyzed using the Kruskal Wallis nonparametric test (PROC NPAR1WAY, SAS Institute Inc., 2004) with group as class variable. Percentage of early apoptotic and late apoptotic/necrotic cells were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with group as class variable. Egg was the smallest experimental unit.

Percentage of egg weight loss during incubation, total percentage of egg weight loss, albumen pH at 18, 42, 66, and 90 hours of incubation, fertility, hatchability of set eggs, hatchability of fertile eggs, embryonic mortality, percentage of second-grade chicks, incubation duration, body weight, chick length, yolk-free body mass, residual yolk weight, organ weights, and navel

quality were analyzed using general linear regression (PROC GLM, SAS Institute Inc., 2004) with PSI, FW, HI, and their interactions as class variables. For analysis of all just mentioned variables, except for albumen pH at 18, 42, 66, and 90 hours of incubation, setter tray was the smallest experimental unit. For analysis of albumen pH at 18, 42, 66, and 90 hours of incubation, egg was the smallest experimental unit. An arcsine transformation was used for embryonic mortality from days 18 to 19 of incubation to obtain normal distributed data. Average fresh egg weight was introduced as a covariate when body weight, chick length, yolk-free body mass, and residual yolk weight were analyzed. Yolk-free body mass was introduced as a covariate when organ weights were analyzed. Values are expressed as least squares means ± SEM. Least squares means were compared using Bonferroni adjustments for multiple comparisons. All main factors and interactions were analyzed for significance at $P \leq 0.05$. Interactions were stepwise excluded from the model when $P > 0.05$. The stage of embryonic development at the end of storage was analyzed using logistic regression analysis (PROC LOGISTIC, SAS Institute Inc., 2004) with PSI, FW, day of analysis, and their interactions as class variables. The stage of embryonic development at 18, 42, 66, and 90 hours of incubation was analyzed using logistic regression analysis (PROC LOGISTIC, SAS Institute Inc., 2004) with PSI, FW, HI, and their interactions as class variables. Egg was the smallest experimental unit. All main factors and interactions were analyzed for significance at $P \leq 0.05$. Interactions were stepwise excluded from the model when $P > 0.05$.

Results

Egg Weight and Egg Weight Loss

An interaction for the average egg weigth was observed between FW and HI ($P = 0.04$), but multiple comparisons showed no significant difference (Table 1). No interaction was observed among treatments for egg weight loss. PSI and FW increased egg weight loss during storage by 0.12% ($P < 0.001$ and $P < 0.001$, respectively). PSI did not affect egg weight loss during incubation and total egg weight loss. However, FW increased egg weight loss during incubation by 0.28% and total egg weight loss by 0.39% ($P < 0.001$ and $P < 0.001$, respectively). HI decreased egg weight loss during incubation by 0.14% ($P = 0.05$).

Table 1. Fresh egg weights and the effect of prestorage incubation (PSI), frequent warming during storage (FW), and hypercapnic incubation (HI) on egg weight loss during storage, incubation, and total egg weight loss

Treatment	n[1]	Fresh egg weight, g	Egg weight loss during storage, %	Egg weight loss during incubation, %	Total egg weight loss, %
PSI					
No	32	66.90	1.02[b]	10.43	11.45
Yes	32	67.04	1.14[a]	10.35	11.50
SEM		0.10	0.02	0.05	0.05
FW					
No	32	66.95	1.02[b]	10.25[b]	11.28[b]
Yes	32	67.00	1.14[a]	10.53[a]	11.67[a]
SEM		0.10	0.02	0.05	0.05
HI					
No	32	67.01		10.46[a]	11.54
Yes	32	66.93		10.32[b]	11.41
SEM		0.10		0.05	0.05
FW x HI					
No x No	16	67.13			
No x Yes	16	66.76			
Yes x No	16	66.89			
Yes x Yes	16	67.10			
SEM		0.14			
***P*-value**					
PSI		0.33	<0.001	0.29	0.48
FW		0.72	<0.001	<0.001	<0.001
HI		0.59		0.05	0.09
PSI x FW		ns	ns	ns	ns
PSI x HI		ns		ns	ns
FW x HI		0.04		ns	ns
PSI x FW x HI		ns		ns	ns

[a-b]Least squares means within a column and treatment lacking a common superscript differ ($P \leq 0.05$). [1]n = number of setter trays that contained 150 eggs at the start of the experiment.

Embryo Characteristics before Incubation

During storage, the total number of embryonic cells decreased from 49,317 on the first day after oviposition to 36,198 at the end of storage (control treatment; $P < 0.001$; Table 2). At the end of storage, the total number of embryonic cells in the PSI and FW treatments (54,303 and 52,458, respectively) were not different from the total number of embryonic cells on the first day after oviposition (49,317), but were higher than the total number of embryonic cells in the control treatment at the end of storage (36,198). The total number of embryonic cells in the PSI plus FW warming treatment (84,577) was higher than in all other treatments. Comparable differences were observed for the number of viable embryonic cells ($P < 0.001$), except that the number of viable embryonic cells on the first day after oviposition (29,930) was comparable to number of viable embryonic cells in the control treatment at the end of storage (30,826).
On the first day after oviposition, the percentage of early apoptotic cells (38.6%) was higher than in all treatments at the end of storage (11.0%, 11.0%, 8.9%, and 8.8%, for the control, PSI, FW, and PSI plus FW treatment, respectively; $P < 0.001$).

On the first day after oviposition, the percentage of late apoptotic/necrotic cells was 1.6%. This percentage was comparable to the percentage of late apoptotic/necrotic cells in the control (3.9%), PSI (5.7%), and FW (4.6%) treatments at the end of storage. Percentage of apoptotic/necrotic cells on the first day after oviposition and in the control treatment at the end of storage was lower than in the PSI plus FW treatment at the end of storage (8.6%; $P = 0.005$).

Table 2. The number of embryonic cells (total and viable embryonic cells), percentage of early apoptotic, and late apoptotic/necrotic cells on the first day after oviposition and at the end of storage and the effect of prestorage incubation (PSI) and frequent warming during storage (FW) on the number of embryonic cells (total and viable embryonic cells), percentage of early apoptotic, and late apoptotic/necrotic cells at the end of storage

Treatment	Group[1]	Total number of embryonic cells	Number of viable embryonic cells	Cells in early apoptosis, %	Cells in late apoptosis/ necrosis, %
First day after oviposition	1	49,317^{b}	29,930^{c}	38.6^{a}	1.6^{b}
End of storage					
PSI x FW					
No x No	2	36,198^{c}	30,826^{c}	11.0^{b}	3.9^{b}
Yes x No	3	54,303^{b}	45,493^{b}	11.0^{b}	5.7^{ab}
No x Yes	4	52,458^{b}	45,818^{b}	8.9^{b}	4.6^{ab}
Yes x Yes	5	84,577^{a}	70,999^{a}	8.8^{b}	8.6^{a}
SEM				2.6	1.2
n		47	46	51	51
***P*-value**		<0.001	<0.001	<0.001	0.005

$^{a\text{-}c}$Means (total and viable embryonic cells) and least squares means (early apoptotic and late apoptotic/necrotic cells) within a column lacking a common superscript differ ($P \leq 0.05$). [1]Group was introduced as class variable in the statistical analysis.

On the first day after oviposition, 3.4% of the embryos was at developmental stage EG8, 20.7% of the embryos was at developmental stage EG9, 48.3% of the embryos was at developmental stage EG10, 17.2% of the embryos was at developmental stage EG11, and 10.3% of the embryos was at developmental stage EG12.

The stage of embryonic development was different between the two days of analysis at the end of storage (P = 0,004), but no interactions with PSI or FW were observed. At the end of storage, also no interaction was observed between PSI and FW for the stage of embryonic development. PSI and FW both increased the stage of embryonic development ($P < 0.001$ and $P < 0.001$, respectively). The change in the stage of embryonic development due to the PSI and FW treatments is shown in Figure 1.

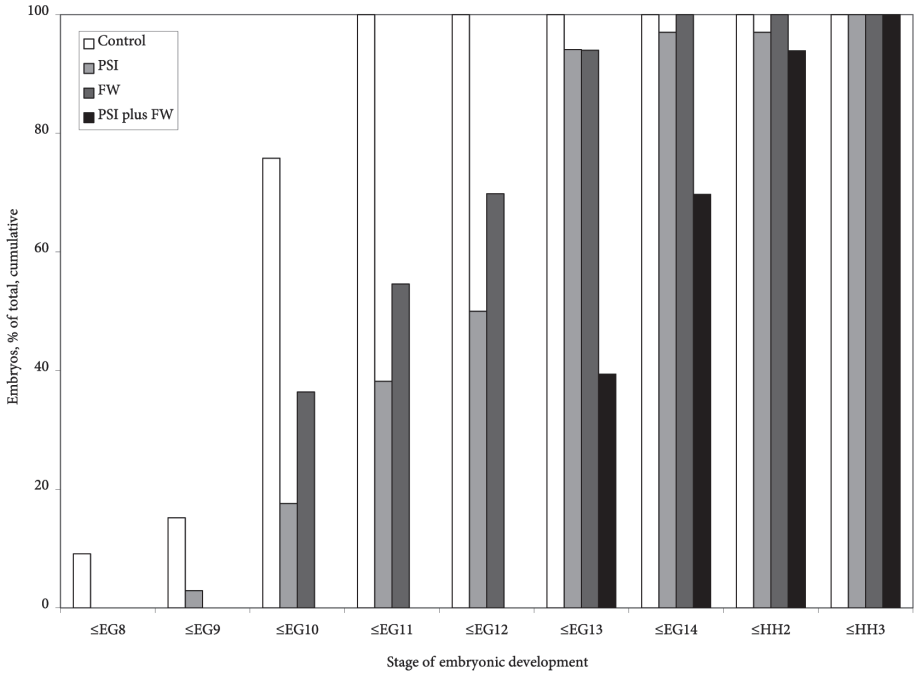

Figure 1. The effect of prestorage incubation (PSI) and frequent warming during storage (FW) on the stage of embryonic development at the end of storage.

Egg Characteristics during Storage and Early Incubation

On the first day after oviposition, albumen height was 7.67 mm, albumen pH was 8.61, and yolk pH was 6.12. No interactions were observed among treatments for egg characteristics at the end of storage and during early incubation (Table 3). PSI resulted in a higher yolk pH (Δ=0.05) at the end of storage ($P = 0.001$), but did not affect albumen height or albumen pH. FW resulted in a higher albumen height (Δ=0.31 mm) and a lower albumen pH (Δ=0.05) at the end of storage ($P = 0.04$ and $P < 0.001$, respectively), but did not affect yolk pH. PSI did not affect albumen pH during the first 90 hours of incubation. FW resulted in a lower albumen pH at 66 hours of incubation (Δ=0.04; $P = 0.003$), but did not affect albumen pH at 18, 42, and 90 hours of incubation. HI decreased albumen pH by 0.34 at 18 hours of incubation, by 0.46 at 42 hours of incubation, by 0.45 at 66 hours or incubation, and by 0.28 at 90 hours of incubation (all $P < 0.001$).

Table 3. Effect of prestorage incubation (PSI) and frequent warming during storage (FW) on egg characteristics at the end of storage and the effect of PSI, FW, and hypercapnic incubation (HI) on egg characteristics during the first 4 days of incubation

	Albumen height, mm	Albumen pH	Yolk pH	Albumen pH	Albumen pH	Albumen pH	Albumen pH
Treatment	**End of storage**	**End of storage**	**End of storage**	**18 hours of incubation**	**42 hours of incubation**	**66 hours of incubation**	**90 hours of incubation**
PSI							
No	4.59	9.42	6.18^{b}	9.28	9.07	8.95	8.69
Yes	4.78	9.43	6.23^{a}	9.28	9.07	8.93	8.66
SEM	0.10	0.01	0.01	0.01	0.01	0.01	0.02
FW							
No	4.53^{b}	9.45^{a}	6.20	9.29	9.07	8.96^{a}	8.69
Yes	4.84^{a}	9.40^{b}	6.21	9.28	9.07	8.92^{b}	8.65
SEM	0.10	0.01	0.01	0.01	0.01	0.01	0.02
HI							
No				9.45^{a}	9.30^{a}	9.17^{a}	8.81^{a}
Yes				9.11^{b}	8.84^{b}	8.72^{b}	8.53^{b}
SEM				0.01	0.01	0.01	0.02
n	152	142	135	119	102	105	86
***P*-value**							
PSI	0.20	0.87	0.001	0.46	0.64	0.10	0.38
FW	0.04	<0.001	0.61	0.23	0.60	0.003	0.35
HI				<0.001	<0.001	<0.001	<0.001
PSI x FW	ns	ns	ns	ns	ns	ns	ns
PSI x HI				ns	ns	ns	ns
FW x HI				ns	ns	ns	ns
PSI x FW x HI				ns	ns	ns	ns

$^{a\text{-}b}$Least squares means within a column and treatment lacking a common superscript differ ($P \leq 0.05$).

Embryonic Development during Incubation

No interactions were observed among treatments for embryonic development during incubation. The FW treatment advanced the stage of embryonic development at 18 hours of incubation ($P < 0.001$) in comparison to the control treatment (Figure 2). At 42, 66, and 90 hours of incubation, embryos of the PSI and FW treatments were both further developed than embryos of the control treatment ($P \leq 0.05$). Effects of the PSI and FW treatments on the stage of embryonic development at 18 and 90 hours of incubation are shown in Figure 2 and 3, respectively.

HI did not affect the stage of embryonic development at 18, 42, 66, and 90 hours of incubation. However, HI tended to retard embryonic development between 66 hours and 90 hours of incubation ($P = 0.08$; Figure 4).

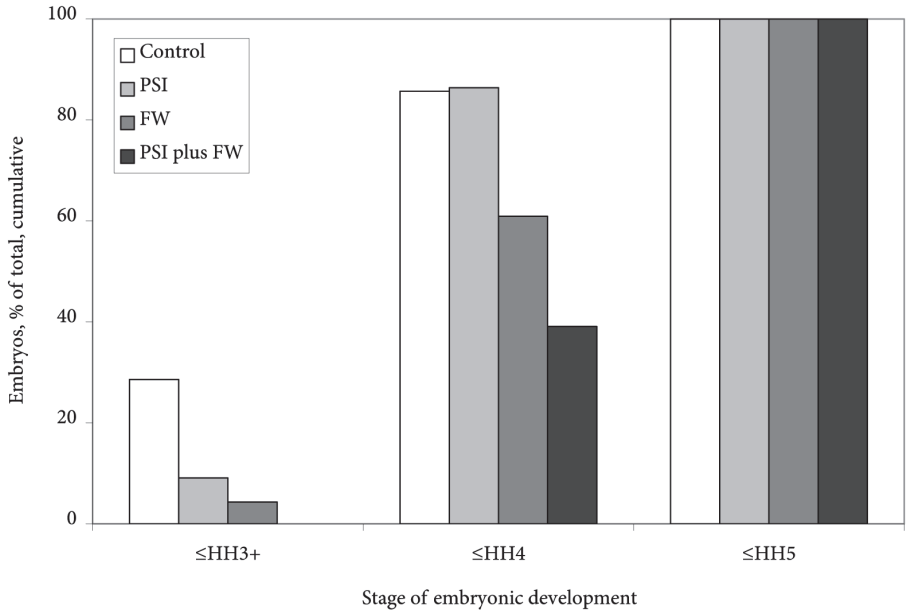

Figure 2. The effect of prestorage incubation (PSI) and frequent warming during storage (FW) on the stage of embryonic development at 18 hours of incubation.

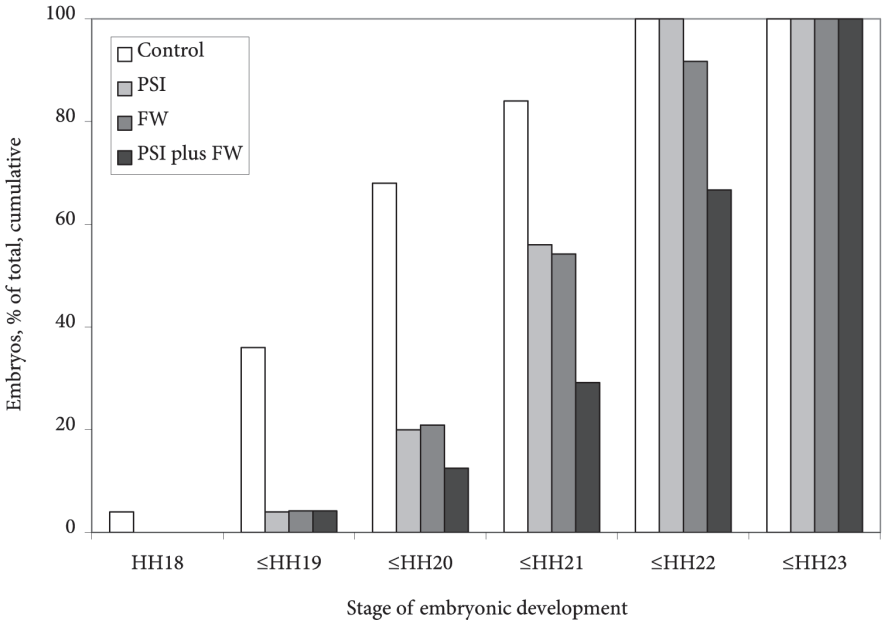

Figure 3. The effect of prestorage incubation (PSI) and frequent warming during storage (FW) on the stage of embryonic development at 90 hours of incubation.

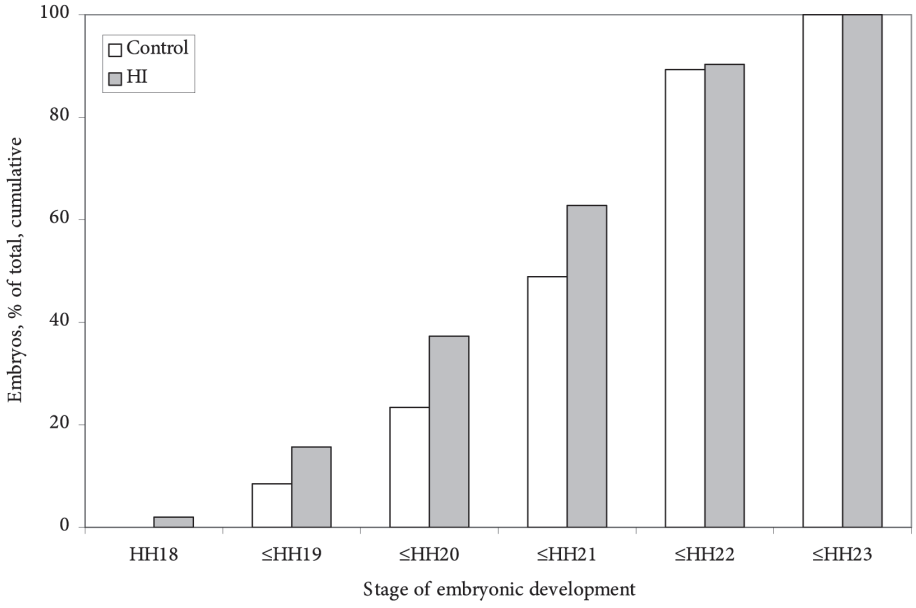

Figure 4. The effect of hypercapnic incubation (HI) on the stage of embryonic development at 90 hours of incubation.

Incubation Duration

No interactions were observed among the treatments for incubation duration. PSI decreased incubation duration by 4 hours (499 hours vs. 495 hours; $P < 0.001$). FW decreased incubation duration by 5 hours (500 hours vs. 495 hours; $P < 0.001$). HI increased incubation duration by 2 hours (496 hours vs. 498 hours ; $P = 0.003$).

Fertility, Embryonic Mortality, and Hatchability

The PSI plus FW treatment had a 2.5% lower hatchability of fertile eggs than the PSI treatment. Hatchability of fertile eggs did not differ among the other treatments (interaction PSI x FW; $P = 0.006$; Table 4). An interaction between PSI and FW was also observed for embryonic mortality from days 4 to 9 of incubation ($P = 0.002$), but multiple comparisons showed no significant difference. PSI decreased embryonic mortality during the first 2 days of incubation by 0.8% ($P = 0.01$) and decreased embryonic mortality on day 20 of incubation by 0.3% ($P = 0.05$). FW increased percentage of malpositioned embryos by 0.5% ($P = 0.02$).

Percentage of eggs classified as infertile was 1.2% lower in the HI treatment than in the control treatment ($P = 0.02$). HI increased embryonic mortality from days 4 to 9 of incubation by 0.6% ($P = 0.03$) and from days 18 to 19 of incubation by 0.4% ($P = 0.04$) and decreased hatchability of fertile eggs by 1.3% ($P = 0.04$). HI also increased percentage of malpositioned embryos by 0.8% ($P < 0.001$).

Table 4. Effect of prestorage incubation (PSI), frequent warming during storage (FW), and hypercapnic incubation (HI) on fertility, hatchability, embryonic mortality, malformationed embryos, malpositioned embryos, and percentage of second-grade chicks

					Embryonic mortality, % of fertile eggs										
Treatment	**n[1]**	**Fertility, %**	**Hatchability of set eggs, %**	**Hatchability of fertile eggs, %**	**d 1 to 2**	**d 3**	**d 4 to 9**	**d 10 to 17**	**d 18 to 19, arcsin**	**d 18 to 19[2]**	**d 20**	**d 21**	**Malformations[3,4], %**	**Malpositions[3,4], %**	**Second-grade chicks[5], %**
PSI															
No	32	95.8	86.2	90.0	2.5[a]	2.9	1.7	0.6	0.07	0.5	0.7[a]	0.4	0.5	1.0	0.3
Yes	32	95.2	86.2	90.5	1.7[b]	3.2	1.6	0.8	0.08	0.6	0.4[b]	0.5	0.4	0.9	0.1
SEM		0.3	0.6	0.4	0.2	0.3	0.2	0.1	0.01		0.1	0.1	0.1	0.1	0.1
FW															
No	32	95.7	86.7	90.6	2.3	2.8	1.7	0.7	0.06	0.4	0.4	0.5	0.4	0.7[b]	0.1
Yes	32	95.3	85.7	89.9	1.9	3.3	1.7	0.7	0.09	0.8	0.7	0.4	0.5	1.2[a]	0.3
SEM		0.3	0.6	0.4	0.2	0.3	0.2	0.1	0.01		0.1	0.1	0.1	0.1	0.1
HI															
No	32	94.9[b]	86.3	90.9[a]	2.2	2.8	1.4[b]	0.7	0.06[b]	0.4	0.4	0.5	0.4	0.6[b]	0.2
Yes	32	96.1[a]	86.1	89.6[b]	1.9	3.2	2.0[a]	0.7	0.09[a]	0.8	0.7	0.4	0.4	1.4[a]	0.2
SEM		0.3	0.6	0.4	0.2	0.3	0.2	0.1	0.01		0.1	0.1	0.1	0.1	0.1
PSI x FW															
No x No	16			89.4[ab]			2.2								
Yes x No	16			91.8[a]			1.2								
No x Yes	16			90.5[ab]			1.3								
Yes x Yes	16			89.3[b]			2.1								
SEM				0.6			0.3								
***P*-value**															
PSI		0.29	0.91	0.39	0.01	0.32	0.72	0.19	0.54		0.05	0.37	0.75	0.56	0.11
FW		0.35	0.20	0.30	0.19	0.14	0.95	0.80	0.08		0.07	0.34	0.40	0.02	0.08
HI		0.02	0.74	0.04	0.38	0.27	0.03	0.98	0.04		0.08	0.73	0.99	<0.001	0.57
PSI x FW		ns	ns	0.006	ns	ns	0.002	ns	ns		ns	ns	ns	ns	ns
PSI x HI		ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns
FW x HI		ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns
PSI x FW x HI		ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns

[a-b]Least squares means within a column and treatment lacking a common superscript differ ($P \leq 0.05$). [1]n = number of setter trays that contained 150 eggs at the start of the experiment. [2]Transformed back to original least squares means. [3]Number of malformationed and malpositioned embryos as a percentage of fertile eggs. [4]The malpositioned and malformationed embryos were already classified as described by Reijrink et al. (2009) and, therefore, the malpositioned and malformationed embryos are double counts. [5]Number of second-grade chicks as a percentage of total number of hatched chicks.

Chick Quality

No interactions were observed among treatments for chick quality variables. PSI, FW, and HI also did not affect chick quality variables. Average values were 42.6 g for body weight, 19.8 cm for chick length, 38.0 g for yolk-free body mass, 4.63 g for residual yolk weight, 0.34 g for heart weight, 1.12 g for liver weight, 2.30 g for stomach weight, and 1.5 for navel quality score.

Discussion

PSI and FW both resulted in a similar increase in egg weight loss during storage. The increased temperature during PSI and FW probably increased egg weight loss during storage because water vapour pressure increases when temperature increases (Meijerhof and Van Beek, 1993). FW also increased egg weight loss during incubation. This may be caused by hair cracks in the eggshells of some of the eggs, due to the repeated egg handling during storage. HI decreased egg weight loss during incubation by 0.14%, but did not affect total egg weight loss. Because relative humidity and eggshell temperatures were similar among the four incubators, the reason for the effect of HI on egg weight loss during incubation is unclear.

In the current study, total percentage of apoptotic cells was 40.2% (38.6% early apoptotic cells + 1.6% late apoptotic/necrotic cells) on the first day after oviposition. The reason for the high percentage of apoptotic cells is unknown, but Bakst and Akuffo (1999) also observed a reduction of 32.8% in the total number of turkey embryonic cells during the first 2 days of storage. They hypothesized that this cell loss was part of the normal morphogenetic development of embryos post ovipostion. Kochav et al. (1980) showed that during the formation of the area pellucida, one-fifth of the total number of cells disappeared from the embryo. At oviposition, turkey embryos show the first signs of area pellucida formation, but most chicken embryos already completed the area pellucida (stage EG10) (Bakst et al., 1997). The high percentage of cell death observed in turkey embryos in the study of Bakst and Akuffo (1999) may, therefore, indeed be part of the normal morphogenetic development after oviposition. In the current study, the high percentage of early apoptotic cells may be partly explained by the formation of the area pellucida that was still in progress in some of the embryos at oviposition (24.2% of the embryos was below developmental stage EG10), but there may be another unknown explanation as well.

Bloom et al. (1998) observed only 3.1% apoptotic cells in fresh laid eggs. They only measured the percentage of late apoptotic/necrotic cells and not the percentage of early apoptotic cells or the change in total number of embryonic cells. This may explain why they observed a lower percentage of total cell death than in the current study and in the study of Bakst and Akuffo (1999).

At the end of storage, we observed 11% of early apoptotic cells and 3.9% of late apoptotic/necrotic cells. Bloom et al. (1998), observed 14% of apoptotic cells in eggs stored for 14 days at 12°C. The percentage of apoptotic cells observed in the current study and in the study of Bloom et al. (1998) are likely time dependent because the residuals of the apoptotic cells are probably removed after the cells died (Glücksmann, 1951; Jacobson et al., 1997; Lockshin and Zakeri, 2004). Total cell death is likely higher than the percentage found by Bloom et al. (1998) as can be seen in the reduction of total number of embryonic cells during storage in the current study and in the study of Bakst and Akuffo (1999). During storage, total number of embryonic cells decreased by 26.6% (from 49,317 to 36,198). This percentage of cell loss plus the percentage of early and late apoptotic cells measured at the end of storage was 41.5% and is comparable to the total percentage of cell death found on the first day after oviposition, which was 40.2%.

This suggests that in the current study, cell death did not increase during storage and that there was also no mitotic activity in the embryos. Activity that probably occurred in the embryo was the removal of some of the cells that died soon after oviposition. At the end of storage, some of the cells that died soon after oviposition were probably not removed yet and these cells were probably found as early apoptotic (11.0%) and late apoptotic/necrotic cells (3.9%).

To investigate how many cells died during storage it seems useful to measure the change in total number of embryonic cells between oviposition and the end of storage and the percentage of early apoptotic and late apoptotic/necrotic cells present in the embryo at the moment of measuring. In the current study, cell death did not increase during egg storage and, therefore, the number of viable embryonic cells did not decrease.

In the current study, PSI and FW increased the stage of embryonic development, the total number of embryonic cells, and the total number of viable embryonic cells. It seems that the stage of embryonic development is linked to a certain number of embryonic cells. How far an embryo develops depends on warming duration and temperature. At the end of storage, the stage of embryonic development was comparable for the PSI and the FW treatments, which suggests that the total input of heat was comparable for both treatments. In the PSI treatment, eggs were at a constant temperature of 37.8˚C for 3 hours and in the FW treatment eggs were warmed 6 times for 30 minutes in water at 37.8˚C, which is also 3 hours.

An interaction was observed between PSI and FW for embryonic mortality from days 4 to 9 of incubation. Although multiple comparisons were not significant, embryonic mortality from days 4 to 9 of incubation in the PSI and FW treatments were lower than in the control and PSI plus FW treatments. PSI also decreased embryonic mortality during the first 2 days of incubation, which was in agreement with Reijrink et al. (2009). In conclusion, some small

effects of PSI and FW on embryonic mortality were observed, but hatchability of fertile eggs was not improved due to these treatments. In a study of Fasenko et al. (2001b), the PSI treatment of 6 hours improved hatchability (81.9% vs. 72.2%) in comparison to the control treatment when eggs were stored for 14 days. After their PSI treatment of 6 hours, 76.7% of the embryos were at developmental stage EG13. They hypothesized that embryos at developmental stage EG13 are less sensitive to prolonged egg storage than embryos that are less or further advanced. In the current study, the PSI treatment had 44.1% and the FW treatment had 24.3% of their embryos at developmental stage EG13 at the end of storage. This may be too less to have a positive effect on hatchability. On the other hand, the stage of embryonic development on the first day after oviposition in the current study was perhaps not highly sensitive to prolonged egg storage. Reijrink et al. (2009) showed that the effect of PSI on hatchability is most beneficial when the majority of the embryos are below developmental stage EG10 at egg collection. In the current study, 24.2% of the embryos was below developmental stage EG10 at egg collection. This percentage may be too low to find a positive effect of PSI or FW on hatchability. In the current study, hatchability was also relatively high in comparison to hatchability results of other studies in which egg storage was prolonged (Fasenko et al., 2001b; Reijrink et al., 2009). This suggests that the age of the breeder flock and/or strain that was used in the current study positively affected embryo viability and the sensitivity of the embryo to prolonged egg storage. PSI and FW were probably not necesarry to make the embryos more resistant to prolonged egg storage and did, therefore, not improve hatchability. The largest effect of the PSI and FW treatments was that they increased embryonic development and decreased incubation duration.

HI decreased albumen pH to 9.11 vs. 9.45 at 18 hours of incubation and the percentage of eggs that was classified as infertile decreased by 1.2%. In the current study, fertility was determined macroscopically and consequently some eggs that were classified as infertile eggs may have been fertile eggs that contained a dead embryo. This suggests that HI was related to the number of embryos that continued their development at the onset of incubation. Gildersleeve and Boeschen (1983) also observed a decrease in turkey embryonic mortality from days 0 to 4 of incubation when CO_2 was injected to a concentration of 0.30% from the onset of incubation until day 10 of incubation. The positive effect of HI on embryo survival during early incubation may be related to the decreased albumen pH. However, the albumen pH of 9.11 at 18 hours of incubation was not close to the optimal pH for embryonic development, which is in the range of 7.9 to 8.4 according to Gillespie and McHanwell (1987). Although total albumen pH was 9.11 at 18 hours of incubation, the pH in the micro-environment of the embryo may have been closer to the range of 7.9 to 8.4 because CO_2 production of the embryo itself may have decreased the pH close to the embryo.

On the other hand, HI retarded embryonic development after 66 hours of incubation and increased embryonic mortality from days 4 to 9 of incubation and from days 18 to 19 of incubation, which resulted in a lower hatchability of fertile eggs. These results suggest that embryos became more sensitive to a high CO_2 concentration after 66 hours of incubation and that we maintained the HI treatment too long. Sadler et al. (1954) showed that the tolerance for CO_2 is at least 4.0% during the first 48 hours of incubation and 1.0% between 48 hours and 72 hours of incubation. In addition, Taylor et al. (1956) showed that any concentration of CO_2 below 1.0% during the first 96 hours of incubation did not affect hatchability. In another study, Taylor and Kreutziger (1965) showed that CO_2 concentrations below 3.0% did not affect hatchability from days 5 to 8 of incubation. Because the CO_2 concentration in the current study was below 1.0% during the first 5 days of incubation, it is unclear why a lower hatchability was found for the HI treatment. Meuer et al. (1989) showed that a CO_2 concentration of 3.0% outside the egg decreased the blood and interstitial pH values of 4 and 6-days-old chicken embryos. They hypothesized that this decrease in pH, due to the increase in the outside CO_2 concentration, could reduce or inhibit mitosis during early embryonic development. In the current study, the CO_2 concentration was not as high as in the study of Meuer et al. (1989), but we may have observed a negative effect on embryonic development and hatchability with a lower CO_2 concentration because mitosis in the embryo was already reduced due to prolonged egg storage (Singal and Kosin, 1969). When mitosis is lower due to prolonged egg storage, a further reduction of mitosis, due to an increased CO_2 concentration in the incubator, may cause abnormal development and/or embryonic mortality at concentrations lower than the one shown by Meuer et al. (1989).

In the current study, HI increased percentage of malpositioned embryos by 0.8%. Gildersleeve and Boeschen (1983) also showed that CO_2 had a long lasting effect on embryo orientation. They observed a reduction in the number of malpositioned embryos when CO_2 concentration was maintained at 0.30% instead of at 0.10% from onset of incubation until day 10 of incubation. However, they observed an increase in the number of malpositioned embryos when CO_2 was kept at 0.30% instead of at 0.10% from onset of incubation until day 14 of incubation. The cause for this long lasting effect of CO_2 is unknown.

One of the hypotheses of the current study was that the effect of HI on hatchability and chick quality depends on the stage of embryonic development at the onset of incubation. An embryo that increased development during storage by PSI or FW may be better able to form an effective barrier between the inside of the embryo and its exterior than a less developed embryo. It is also possible that a more developed embryo decreases the pH in its micro-environment close

to the optimal range of 7.9 to 8.4 due to its own CO_2 production. When these hypotheses were true, we expected that a decrease in albumen pH due to HI would not be necessary when the embryos were more developed at the onset of incubation due to PSI or FW. However, we did not observe an interaction between neither PSI and HI nor between FW and HI for fertility, hatchability, or chick quality. This suggests that the effect of HI did not depend on the stage of embryonic development at the onset of incubation.

In the current study, none of the treatments had an affect on chick quality on the day of hatch in terms of chick length or yolk-free body mass. In the study of Reijrink et al. (2009), PSI also did not affect chick length on the day of hatch when egg storage was prolonged. In studies of Bruggeman et al. (2007) and De Smit et al. (2006, 2008), a gradual increase of CO_2 during the first 10 days of incubation to a concentration of 1.0% to 1.5% also did not affect body weight on the day of hatch.

In conclusion, PSI and FW increases the stage of embryonic development and the number of viable embryonic cells. Consequently, incubation duration decreases, but PSI and FW do not affect hatchability or chick quality. HI decreases pH of the total albumen, which may relate to the increase in the number of embryos that continues their development at the onset of incubation. However, because HI also retards embryonic development and decreases hatchability, HI at concentrations between 0.70% to 0.80% during the first 5 days of incubation is probably too long.

Acknowledgments

We would like to thank Marcel Heetkamp, Ilona van den Anker, Sonja de Vries, Ewoud Hazejager (Adaptation Physiology Group, Wageningen University, Wageningen, the Netherlands), Anja Taverne-Thiele (Cell Biology and Immunology Group, Wageningen University, Wageningen, the Netherlands), Woutrien de Bruin, Conny Maatjens, Roos Molenaar, Cor Termaat, Willem van den Oetelaar, and Stefan Achterberg (HatchTech Incubation Technology B.V., Veenendaal, the Netherlands) for their assistance during the experiment. We also would like to thank Henri Woelders (Animal Sciences Group, Lelystad, the Netherlands), Henny van Straaten (Department of Anatomy and Embryology, University of Maastricht, Maastricht, the Netherlands), Jacob Hamidu (Department of Food and Nutritional Science, University of Alberta, Alberta, Canada), and Makhsud Tagirov (Poultry Research Institute, Ukrainian Academy of Agrarian Sciences, Birky, Ukraine) for their assistance by optimizing the method to dissociate embryonic cells. We are also grateful to Lagerwey hatchery (Lagerwey B.V., Lunteren, the Netherlands) and the Knoops family (Breeder Farm, Someren, the Netherlands) for their cooperation.

Chapter 6

General Discussion

Introduction

Storage of hatching eggs is a common procedure at breeder farms and hatcheries. Normally, commercial hatcheries set their eggs after 3 to 5 days of storage to minimize the negative effects of egg storage on hatchability and chick quality. However, due to variations in the supply of hatching eggs and the market demand for day-old chicks, hatcheries sometimes need to increase the storage duration. It is well known that an increase in storage duration increases hatch time (Mather and Laughlin, 1976; Tona et al., 2003), decreases hatchability (Becker, 1964; Merritt, 1964; Fasenko et al., 2001b; Tona et al., 2004; Yassin et al., 2008), decreases chick quality on the day of hatch (Byng and Nash, 1962; Tona et al., 2003, 2004), decreases subsequent performance (Becker, 1960; Merritt, 1964; Tona et al., 2003, 2004), and increases posthatch mortality (Merritt, 1964; Yassin et al., 2009). A storage duration beyond 7 days has a pronounced effect on hatchability (Yassin et al., 2008). On average, each extra day of storage up to the seventh day of storage reduces hatchability by 0.2%, while this percentage increases to 0.5% after the seventh day of storage (Yassin et al., 2008). In the current thesis, the control treatments of Chapter 2 and 4 showed that hatchability decreased by about 0.93% per storage day when storage duration increased from 3 to 14 days. This decrease in hatchability was mainly caused by an increase in embryonic mortality from days 0 to 9 of incubation (Figure 1). A negative effect from prolonged egg storage on chick quality was also observed. Periods of 4 and 13 or 14 days of storage were compared. Yolk-free body mass on the day of hatch decreased by 0.5 g, chick length on the day of hatch decreased by 0.1 cm, and the percentage of second-grade chicks increased by 0.6% (Chapter 4).

The reasons for the negative effects of prolonged egg storage on hatchability and chick quality are unclear. These negative effects may be caused by changes in the embryo, changes in the egg characteristics (micro-environment of the embryo), or by both (Becker et al., 1968; Meijerhof, 1992; Chapter 1).

The primary aim of the current thesis was to investigate which physiological mechanisms are involved in the negative effects of prolonged egg storage on hatchability and chick quality. The second aim of the current thesis was to investigate how negative effects of prolonged egg storage on hatchability and chick quality can be reduced by making changes in the storage or early incubation conditions. In Chapter 1, we hypothesized that the risk of the embryo to die during incubation after prolonged egg storage likely depends on the stage of embryonic development, the number of viable embryonic cells, and the micro-environment of the embryo during storage and/or early incubation.

In the current chapter, it is discussed whether this hypothesis is true based on the results of Chapters 2, 3, 4, and 5. Firstly, it is discussed whether the stage of embryonic development determines the sensitivity of the embryo to prolonged egg storage. Secondly, the involvement of the number of viable embryonic cells is reflected. The involvement of the micro-environment

of the embryo during storage and early incubation are then discussed, followed by the effects of prolonged egg storage on chick quality. Finally, the conclusions of the current thesis are presented.

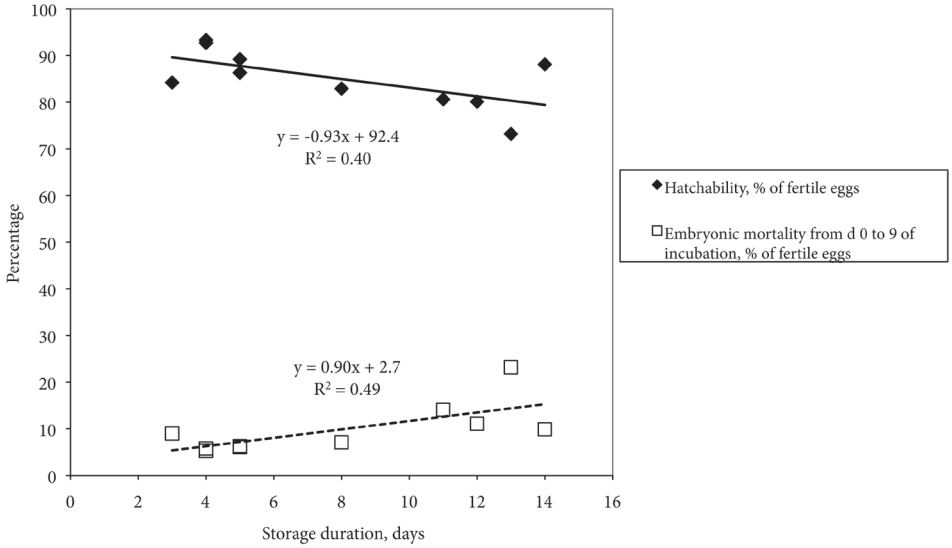

Figure 1. Effect of storage duration on embryonic mortality from days 0 to 9 of incubation as a percentage of fertile eggs and hatchability as a percentage of fertile eggs (based on the control treatments of Chapter 2 and 4).

Stage of Embryonic Development

Several authors have compared the stages of embryonic development from flocks with different hatchability results. These authors have suggested that the stage of embryonic development at oviposition is related to hatchability (Hays and Nicolaides, 1934; Coleman and Siegel, 1966; Kosin and Arora, 1966; Steinke, 1972). The sensitivity of an embryo to prolonged egg storage also seems to depend on the stage of embryonic development (Kosin, 1956; Fasenko et al., 2001b). Fasenko et al. (2001b) showed that a prestorage incubation treatment of 6 hours improved hatchability in comparison to the control treatment when storage duration was 14 days, and they linked this improvement to changes in the stage of embryonic development caused by prestorage incubation. After the prestorage incubation treatment, the majority of the embryos were at developmental stage EG13 (Eyal-Giladi and Kochav, 1976 (**EG**)). Fasenko et al. (2001b) hypothesized that embryos at developmental stage EG13 are better able to withstand prolonged egg storage than embryos that are less or further advanced. An embryo

that is advanced beyond developmental stage EG13, is probably more sensitive to prolonged egg storage than an embryo at developmental stage EG13 because it is forming the primitive streak. The formation of the primitive streak is associated with an extremely active period of cellular migration and differentiation (Bellairs, 1986). Storage of eggs in this active period may disturb normal embryonic development.

In the current thesis, it was suggested that an embryo below developmental stage EG10 is also sensitive to prolonged egg storage (Chapter 2). The hypothesis of Fasenko et al. (2001b) and Chapter 2 suggest that an embryo at a developmental stage below EG10 is more sensitive to prolonged egg storage than embryos at stages EG10, EG11, EG12, and EG13. In addition, an embryo at developmental stage EG13 is more resistant to prolonged egg storage than embryos at the developmental stages EG10, EG11, and EG12. The reason why these developmental stages differ in their sensitivity to prolonged egg storage is unknown, but may be explained in two ways.

The area pellucida is formed between developmental stages EG7 and EG10 (Eyal-Giladi and Kochav, 1976). At the same time the prenucleoli mature to nucleoli, which are able to produce mRNA (Raveh et al., 1976). The formation of the area pellucida and the maturation of the nucleoli likely involve enhanced metabolic activity and a wave of glycogen degradation (Eyal-Giladi et al., 1979). The high metabolic activity involved in area pellucida formation may make these embryos less suitable for prolonged egg storage. When embryos are stored during the formation of the area pellucida, it is likely that the glycogen reserves used to maintain crucial processes in embryonic cells are consumed and that insufficient glycogen is left at the onset of incubation to finish the formation of the area pellucida, which may cause embryonic mortality.

Furthermore, differences in organelle differentiation may also play a role in the sensitivity of the embryo to prolonged egg storage (Raveh et al., 1971). Organelles, such as the nucleolus, endoplasmic reticulum, mitochondria, and Golgi complex, are more differentiated in double-layered embryos (embryos that completely formed the hypoblast; EG13) than in single-layered embryos (embryos before hypoblast formation; <EG13). Because embryos differ in organelle differentiation, they may also differ in the ability to produce ATP, which may affect the sensitivity of an embryo to prolonged egg storage.

To investigate whether embryos at a developmental stage below EG10 and above EG13 are more sensitive to prolonged egg storage than embryos in stages EG10, EG11, EG12, and EG13, data from Fasenko et al. (2001b), Chapter 2, Chapter 5, and a pilot study that is not published in the current thesis were combined. In the pilot study, 335 eggs of a Hubbard breeder flock at 48 weeks were used. Eggs were stored for 16 days at 17°C and a relative humidity of 65%. Eggs

were warmed 14 times during storage for 0.5 or 1.5 hours in water at 37.8°C. To compare the data of Fasenko et al. (2001b) with the other data, it was assumed that the embryonic mortality from days 0 to 7 of incubation was comparable to the embryonic mortality from days 0 to 9 of incubation in the experiments of the current thesis and the pilot study. This assumption is validated by the results of Fasenko et al. (2001b) and other studies (Vick et al., 1993; Lourens et al., 2005), which show that the mortality in the second week of incubation is low.

The combined data from Fasenko et al. (2001b), Chapter 2, Chapter 5, and the pilot study contain 15 treatments in total:

- Four treatments from Fasenko et al. (2001b): control (A); 6 hours (B); 12 hours (C); and 18 hours of prestorage incubation (D).
- Four treatments from Chapter 2: two control treatments from experiments I (E) and II (G); 6 hours of prestorage incubation in experiment I (F); and 4.5 hours of prestorage incubation in experiment II (H).
- Four treatments from Chapter 5: control (I); 3 hours of frequent warming during storage (J); 5 hours of prestorage incubation (K); and 5 hours of prestorage incubation + 3 hours of frequent warming during storage (L).
- Three treatments from the pilot study: control (M); 7 hours of frequent warming during storage (N); and 21 hours of frequent warming during storage (O).

To investigate which stage or stages of embryonic development are able to endure prolonged egg storage, it was analyzed whether the risk of mortality during the first 9 days of incubation after prolonged egg storage depends on the stage of embryonic development at the end of storage. Embryonic mortality from days 0 to 9 of incubation was used because a decrease in hatchability due to an increase in storage duration is mainly the result of an increased mortality during this period (Figure 1). Figure 2 shows the embryonic mortality from days 0 to 9 of incubation for the 15 treatments. The 15 treatments were categorized based on the percentage of embryos below developmental stage EG10 or above developmental stage EG13. This categorization was chosen because it was suggested in Chapter 2 that embryos in a developmental stage below EG10 were sensitive to prolonged egg storage and because Fasenko et al. (2001b) suggested that embryos developed beyond stage EG13 were also sensitive to prolonged egg storage. One treatment had no embryos below developmental stage EG10 or higher than EG13 and was placed in between the two above mentioned groups. Figure 2 suggests that embryonic mortality from days 0 to 9 of incubation increases when the majority of the embryos are developed beyond stage EG13.

Embryos developed beyond stage EG13 do not appear often when no prestorage incubation or frequent warming during storage is used. In practice, the number of embryos developed beyond stage EG13 may increase when eggs are not collected consistently in an environmental temperature above 27°C (Fasenko et al., 1991). Development beyond stage EG13 increases embryonic mortality from days 0 to 9 of incubation. Therefore, a long exposure time to high

environmental temperatures and too long periods of prestorage incubation or frequent warming during storage that are too long should be avoided.

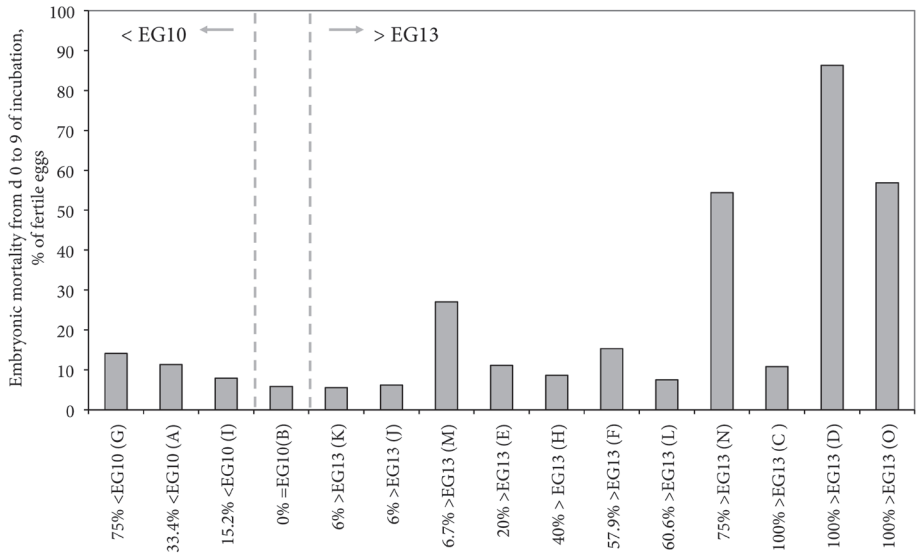

Figure 2. Embryonic mortality from days 0 to 9 of incubation as a percentage of fertile eggs for 15 different treatments (A, B, C, and D from Fasenko et al., 2001b; E, F, G, and H from Chapter 2; I, J, K, and L from Chapter 5; M, N, and O from a pilot study). The treatments are categorized based on the percentage of embryos below developmental stage EG10 and developed beyond stage EG13.

The evidence that embryonic mortality from days 0 to 9 of incubation increases when the majority of the embryos are less developed than stage EG10 is not convincing and should be investigated in more detail. Figure 3 presents the increase or decrease of embryonic mortality from days 0 to 9 of incubation (delta embryonic mortality from days 0 to 9 of incubation) caused by prestorage incubation or frequent warming during storage compared to the control treatment. The order of the different experiments in Figure 3 was chosen based on the developmental stages of the embryos in the control treatments. The experiment with the lowest embryonic development in the control treatment is placed on the left side of the x-axis, whereas the experiment with the highest development in the control treatment is placed on the right side of the x-axis. The other experiments are placed in between the control treatments with the lowest and highest development.

Figure 3 shows that an increase in the stage of embryonic development due to prestorage incubation or frequent warming during storage decreased embryonic mortality from days 0 to 9 of incubation when at least 33.4% of the embryos were in a developmental stage below EG10 at egg collection and when prestorage incubation was not longer than 6 hours.

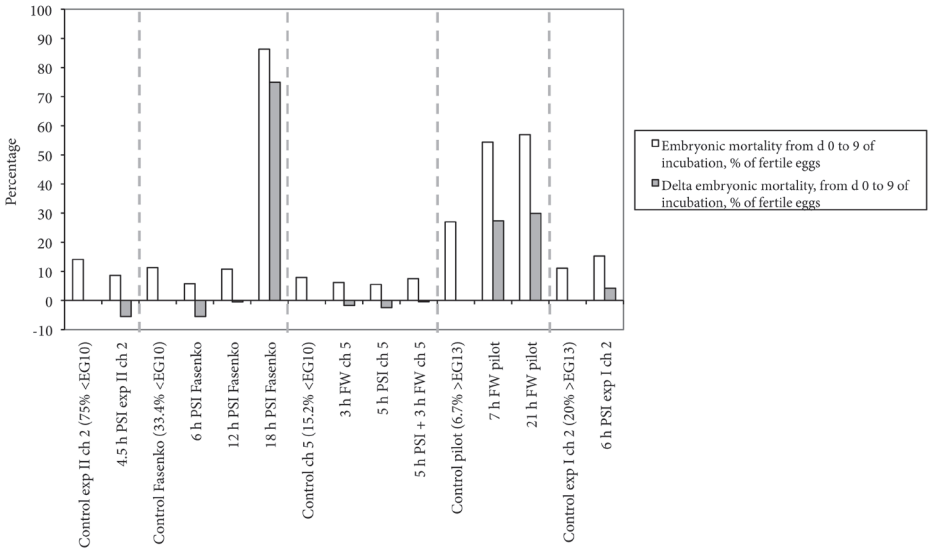

Figure 3. Per experiment (distinguished by a dotted line) effects of prestorage incubation (PSI) or frequent warming during storage (FW) on embryonic mortality from days 0 to 9 of incubation and the increase or decrease in embryonic mortality from days 0 to 9 of incubation (delta embryonic mortality from days 0 to 9 of incubation compared to the control treatment). The order of the different experiments was chosen based on the developmental stage of the embryos of the control treatments (experiment II, Chapter 2; Fasenko et al., 2001b; Chapter 5; pilot study; experiment I, Chapter 2).

The most positive effect of prestorage incubation on hatchability was found when at least 33.4% of the embryos were below stage EG10 at egg collection and when the embryos did not develop beyond stage EG13 due to prestorage incubation. Embryos less developed than stage EG10 occur most often in breeder flocks younger than 30 weeks of age, which may explain why the decrease in hatchability after prolonged egg storage is more pronounced in the eggs of young breeders than old breeders, as shown by Yassin et al. (2008). When eggs of young breeders need to be stored beyond 7 days, the use of prestorage incubation may have a positive effect on hatchability. However, as can be seen in Figure 2, the differences in embryonic mortality from days 0 to 9 of incubation are marginal among the first 11 treatments (G until L), which suggests that embryos can resist prolonged egg storage as long as they are not developed beyond stage

EG13. When embryos are developed beyond stage EG13 due to prestorage incubation or frequent warming during storage, embryonic mortality from days 0 to 9 of incubation can increase to more than 50%. At oviposition, the stage of embryonic development often ranges from EG10 to EG13, which seem to be the stages that are not highly sensitive to prolonged egg storage. Therefore, when prestorage incubation or frequent warming during storage is used to minimize the negative effects of prolonged egg storage, the risk for decreased hatchability is higher than the chance for improvement.

Number of Viable Embryonic Cells

Egg Storage

Arora and Kosin (1967, 1968), Konishi and Kosin (1974), Bloom et al. (1998), and Bakst and Akuffo (1999) showed that cell death in the embryo increases when storage duration increases. This loss of viable embryonic cells may cause the decrease in hatchability after prolonged egg storage. An embryo may need a minimum number of viable cells to continue its development successfully at the onset of incubation. When too many cells die during storage and/or early incubation, particular steps in an embryo's development may be impeded, resulting in abnormal development or embryonic mortality.

In the current thesis, the effect of prolonged egg storage on cell death was only measured in Chapter 5. The total percentage of apoptotic cells was 40.2% on the first day of storage, which resulted mainly from a high percentage of early apoptotic cells (38.6%). Bakst and Akuffo (1999) also observed a reduction of 32.8% in the total number of turkey embryonic cells during the first 2 days of storage. The reason for this high percentage of cell death is unclear, but it may be part of the normal morphogenetic development of embryos after ovipostion (Bakst and Akuffo, 1999). For example, when the area pellucida is formed, one-fifth of the total number of embryonic cells disappears from the embryo (Kochav et al., 1980). Because in Chapter 5, most embryos (75.8%) already completed the formation of the area pellucida at oviposition, there may be a different reason for the high percentage of early apoptotic cells as well. The total cell death on the first day of storage was comparable to the total cell death at the end of storage.

Between the first and last days of storage, the total number of embryonic cells decreased by 26.6% (from 49,317 to 36,198). This percentage of cell loss plus the percentage of early apoptotic (11.0%) and late apoptotic/necrotic cells (3.9%) present in the embryos at the end of storage comes to 41.5%. This total percentage is comparable to the percentage of early apoptotic and late apoptotic/necrotic cells present in the embryo on the first day of storage, which was 40.2%. The number of viable embryonic cells on the first day of storage and at the end of storage, was also comparable (29,930 vs. 30,826). These results suggest that some of the cells that died on the first day of storage were removed during storage. However, some of the apoptotic cells were still

present in the embryo as early apoptotic or late apoptotic/necrotic cells at the end of storage.

When eggs are stored at a temperature around 16°C, the embryo's metabolic rate and energy production are reduced. It is possible, therefore, that the removal of dead cells was retarded and that the dead cells found at the end of storage may have actually died soon after oviposition. These results also suggest that cell death did not increase during storage (but only soon after oviposition) and that no mitotic activity was present in the embryo during storage. The latter is in agreement with results of Arora and Kosin (1967, 1968), who showed that the mitotic cycle was not finished during storage but was blocked at metaphase. Because in Chapter 5 the number of viable embryonic cells was comparable on the first day of storage and at the end of storage, it was not proven that the negative effects of prolonged egg storage are caused by an increase in cell death during storage. However, the removal of dead cells probably consumes energy, and the removal of a high percentage of dead cells may cost stored embryos so much energy at a time when energy production is low, that this may result in embryonic mortality. These energy costs may also be the reason why prolonged egg storage has a negative effect on hatchability.

To investigate whether the number of viable embryonic cells determines embryo viability, it was evaluated whether an increased number of viable embryonic cells resulting from advanced development during storage decreased embryonic mortality and increased hatchability and chick quality (Chapter 5). Although the control and the prestorage incubation plus frequent warming during storage treatments significantly differed in the number of viable embryonic cells at the onset of incubation (30,826 vs. 70,999), hatchability did not differ between these treatments (89.4% vs. 89.3%). The number of viable embryonic cells likely supported the development stages in both treatments and was probably not below the critical threshold needed to continue embryonic development successfully at the onset of incubation. The only pronounced effect of prestorage incubation and frequent warming during storage was the advanced development of the embryos at the onset of incubation, and as a consequence the incubation duration was shorter.

In Chapter 5, the embryonic mortality during the first 9 days of incubation was relatively low (around 7%) for eggs stored beyond 7 days compared to the results of both experiments in Chapter 2 (11.1% in experiment I and 14.1% in experiment II) and experiment II in Chapter 4 (23.2%). This difference may be due to the difference in breeder flock age. The breeder flock age was 36 weeks in Chapter 5, 28 weeks in experiment II of Chapter 2 and 4, and 61 weeks in experiment I of Chapter 2. Witters (2009) showed that the effect of prolonged egg storage on hatchability changed when the breeder flock became older. She stored eggs for 3 or 17 days and observed a decrease in hatchability of 8.1% when the breeder flock was 32 weeks old, 5.5% when the breeder flock was 40 weeks old, 3.6% when the breeder flock was 45 weeks old, and 7.7% when the breeder flock was 58 weeks old. These results suggest that embryos of a breeder

flock of 36 weeks are less sensitive to prolonged egg storage than embryos of a breeder flock of 28 or 61 weeks of age. As stated earlier, this breeder flock age effect may be related to the stage of embryonic development at egg collection.

Number of Viable Embryonic Cells

Preincubation Warming

In Chapter 4, we observed an effect of preincubation warming profile on embryonic mortality from days 0 to 9 of incubation and this effect may involve a change in the number of viable embryonic cells. The 24-h preincubation warming profile improved hatchability compared to the 4-h preincubation warming profile when storage duration was prolonged. We hypothesized that the number of viable embryonic cells increased during the first 10 hours of the 24-h preincubation warming profile due to an increase in mitotic activity before the morphological development continued. Within the first 10 hours of the 24-h preincubation warming profile, the internal egg temperature increased from 17°C to 27°C. Between these temperatures, embryos may not develop morphologically (Funk and Biellier, 1944), but may increase their mitotic activity due to the rise in temperature (Arora and Kosin, 1968). The increase in mitotic activity may have prevented early embryonic mortality for two reasons: 1) The number of viable embryonic cells increased and compensated for the cell death that occurred during storage before the morphological development continued. As a result, the embryos contained enough viable embryonic cells to continue their development successfully at the onset of incubation. 2) Due to the increased mitotic activity, the cells were able to dilute the content of biological 'garbage,' such as damaged macromolecules and organelles (Stroikin et al., 2005; Terman et al., 2006), which may have occurred in the aged cells during storage. The accumulation of 'garbage' is harmful for cell viability because of its toxic effects and because it occupies a certain part of the cell's interior, which interferes with intracellular transport, signaling, and metabolic processes (Stroikin et al., 2005). Consequently, aged cells have a high risk to die. Therefore, it may be beneficial to dilute the content of the biological "garbage" before morphological development continues. Embryos in the 4-h preincubation warming profile continued their development with only aged cells, which may have increased abnormal development and/or embryonic mortality.

Another reason for a reduced embryonic mortality from days 0 to 9 of incubation is that cells may need time to adapt before they can increase their metabolic rate after prolonged egg storage at temperatures below 20°C. Without enough time to adapt before morphological development continues, abnormal development and/or embryonic mortality may increase.
In conclusion, the 24-h preincubation warming profile decreased embryonic mortality from days 0 to 9 of incubation, but the reason why is unclear.

The Micro-Environment of the Embryos during Storage

During egg storage, the albumen pH increases from about 7.6 to about 9.0 within the first 4 days of storage (Lapão et al., 1999), the yolk pH increases from about 6.0 to about 6.5 (Shenstone, 1968), and albumen height decreases. This rise to a pH around 9.0 probably occurs to protect the embryo from microbial contamination (Board and Fuller, 1974). Another reason for the rise in albumen pH may be that the pH gradient between the albumen and yolk is necessary for the orientation of the embryo (the dorso-ventral polarity) (Stern, 1991). The reduction in albumen height is probably necessary for the transport of sufficient O_2 to the embryo during early incubation (Meuer and Baumann, 1988).

Although changes in the egg characteristics seem to be necessary during early incubation, it can also be hypothesized that an albumen pH around 9.0 can have a negative effect on embryo viability when storage is prolonged. Gillespie and McHanwell (1987) investigated the pH in the extracellular space in isolated in vitro chicken embryos at stages varying from 4 to 22 of somites. They showed that during early incubation, the pH of the embryo's extracellular space varied from 7.9 to 8.4 and that the ectodermal and endodermal epithelia form an effective barrier between the inside of the embryo and its exterior (albumen pH around 9.0 and yolk pH around 6.5), which is most likely an active process. An embryo may be unable to maintain the active transport processes needed to maintain an effective barrier during prolonged egg storage because embryonic cells have a limited energy budget. When eggs are stored below temperatures at which morphological development occurs, the embryo's metabolic rate and ATP production are depressed. To maintain homeostasis in any cell, the rate of ATP utilization must match the rate of ATP production (Storey and Storey, 2004). When ATP production is depressed, processes that are not essential for the immediate requirements of the cell, such as macromolecular biosynthesis (protein synthesis, RNA/DNA synthesis), will be suspended for those that are more critical for ionic integrity (Buttgereit and Brand, 1995). Cells are able to maintain an effective barrier between the inside of the embryo and the albumen and yolk, but how long this can be maintained without negative consequences is unknown. When the storage duration is prolonged, the embryo may not have enough energy left to survive during early incubation (Walsh et al., 1995). If this is true, a stabilization of albumen pH within a range of 7.9 to 8.4 from the beginning of a prolonged egg storage duration may decrease embryonic mortality during early incubation because then the maintenance of an effective pH barrier may cost less energy. In Chapter 3, it was shown that the maintenance of albumen pH at 8.70 or 8.48 during storage due to an increase in the CO_2 concentration to 0.74 or 1.50%, respectively, did not affect hatchability and chick quality.

However, several authors improved hatchability when eggs were stored in plastic bags with or without additional N_2 (Becker et al., 1963; Becker, 1964; Proudfoot, 1964a, 1965, Gordon and Siegel, 1966; Becker et al., 1967, 1968; Kosin and Konishi, 1973). Storage of eggs in plastic bags

in which CO_2 and H_2O gradually increased and in which sometimes N_2 gas was added, had a pronounced effect on hatchability when egg storage was beyond 19 days (Proudfoot, 1964a, 1965; Kosin and Konishi, 1973). Due to the gradual increase of CO_2 and H_2O, the albumen pH stabilized at a level somewhere between 7.6 and 9.0, and water loss was decreased in comparison to eggs stored under standard conditions. Because CO_2 and water loss are always affected when eggs are stored in plastic bags, it is difficult to determine what caused the positive effect on hatchability. In Chapter 3, the storage duration was 14 days, which was perhaps too short to find a positive effect of a lower albumen pH on hatchability. An embryo seems capable of maintaining an effective barrier between the inside of the embryo and its exterior (albumen and yolk) for 14 days without negative consequences for survival, but storage beyond 19 days may be too long to maintain the effective barrier.

The positive effect of egg storage in plastic bags on hatchability after 19 days of storage may also be caused by the prevention of water loss. Mayes and Takeballi (1984) and Proudfoot (1969) reviewed the effects of relative humidity during storage on hatchability. Some authors concluded that egg weight loss should be prevented during egg storage and others stated that egg weight loss during storage does not affect hatchability. Therefore, it is still unclear if water loss during storage should be prevented to minimize the negative effects of prolonged egg storage on hatchability. It can be assumed that egg weight loss during storage has no negative effect on hatchability as long as the total egg weight loss during storage and incubation is close to the optimal egg weight loss of 11.5% (Meir and Ar, 1987; Hulet et al., 1987). When egg storage is beyond 14 days, egg weight loss during storage may result in weight losses above 11.5% and may negatively affect hatchability.

In Chapter 3, the 3.0% O_2 concentration did not affect embryonic development, hatchability, and chick quality. A storage temperature of 16°C most likely reduced the metabolism of the embryo to a level that was low enough to survive a storage duration of 14 days. A decrease in the O_2 concentration had no additional effect, but may have had a positive effect when egg storage would have been longer than 14 days.

In conclusion, it is unclear whether the micro-environment of the embryo during storage is involved in the negative effects of prolonged egg storage. However, the results of the current thesis suggest that the role of the micro-environment seems to be minimal when the storage temperature is 16°C and the storage duration is 14 days.

The Micro-environment of the Embryo during Early Incubation

Due to an increase in temperature during early incubation, CO_2 loss from the egg to the environment accelerates. Consequently, the amount of CO_2 in the egg decreases and the albumen pH increases from about 7.6 to about 9.0 within the first 24 hours of incubation when fresh eggs are incubated. The albumen pH stays around 9.0 when stored eggs are incubated (Benton and Brake, 1996). The albumen pH will stay around 9.0 until the CO_2 production of the embryo is sufficient enough to decrease the albumen pH, which occurs after about 72 hours of incubation (Sadler et al., 1954; Cunningham, 1974).

During early incubation, an embryo that has been stored for a prolonged time (> 7 days) may not have enough energy left to maintain an effective barrier between the inside of the embryo and its exterior (albumen pH around 9.0 and yolk pH around 6.5). Insufficient protection of the embryo by the vitelline and chalaziferous membrane during early incubation may enhance the effect of the exterior on the internal pH. It is known that the strength of the yolk membrane decreases during storage (Fromm, 1966). In addition, Sadler (1955) showed that the vitelline and chalaziferous membrane are absent over the area of the embryo before the amnion is completely formed. Consequently, the embryo can be exposed to the alkaline albumen. Prolonged egg storage slows down embryonic development as was shown in Chapter 4. Prolonged egg storage may, therefore, lengthen the time that the embryo is exposed to the alkaline albumen and may increase abnormal development and/or early embryonic mortality.

This problem can perhaps be solved by a high CO_2 concentration in the incubator during early incubation. A high CO_2 concentration in the incubator decreases the albumen pH. When the albumen pH is decreased close to the optimal range of 7.9 to 8.4, the pH gradient across the embryo may still be sufficient and maintenance of the effective barrier will cost less energy. Sadler et al. (1954) also showed that the breakdown of the chalaziferous membrane was retarded when eggs were incubated with increased CO_2 concentrations. These combined factors may prevent abnormal development and/or early embryonic mortality.

In Chapter 5, stored eggs were incubated at CO_2 concentrations between 0.70% and 0.80% during the first 5 days of incubation. The albumen pH decreased from 9.45 to 9.11 after 18 hours of incubation, to 8.84 after 42 hours of incubation, to 8.72 after 66 hours of incubation, and to 8.53 after 90 hours of incubation. The percentage of embryos classified as infertile decreased by 1.2%, which suggests that more embryos were able to continue their development at the onset of incubation than in the control treatment. Although the total albumen pH was 9.11 after 18 hours of incubation, it is possible that the pH close to the embryo was closer to the range of 7.9 to 8.4 measured by Gillespie and McHanwell (1987). The CO_2 production of the embryo increases when embryonic development continues. In addition, resistance to the escape of CO_2

from the albumen is greater when the CO_2 concentration in the incubator increases, which may decrease the pH close to the embryo. This may explain why more embryos were able to continue their development at the onset of incubation even though total albumen pH was 9.11 after 18 hours of incubation, which is above the range of 7.9 to 8.4.

In contrast, CO_2 concentrations between 0.70% and 0.80% during the first 5 days of incubation retarded embryonic development and increased embryonic mortality from days 4 to 9 of incubation and from days 18 to 19 of incubation. An embryo should be tolerant to CO_2 concentrations below 1.0% during the first 96 hours of incubation (Sadler et al., 1954; Taylor et al., 1956) and below 3.0% from days 5 to 8 of incubation (Taylor and Kreutziger, 1965). Therefore, it is unclear why CO_2 concentrations between 0.70% and 0.80% had these negative effects.

Meuer et al. (1989) showed that a CO_2 concentration of 3.0% outside the egg decreased the blood and interstitial pH values of 4 and 6-days-old chicken embryos. They hypothesized that this decrease in pH, due to the increase in the outside CO_2 concentration, could reduce or inhibit mitosis during early embryonic development. In the current study, the CO_2 concentration was not as high as in the study of Meuer et al. (1989). However, it is possible that we observed a negative effect on embryonic development and hatchability with a lower CO_2 concentration because the mitosis in the embryo was already reduced due to prolonged egg storage (Singal and Kosin, 1969). When mitosis is already lower due to prolonged egg storage, a further reduction of mitosis due to an increased CO_2 concentration in the incubator may cause abnormal development and/or embryonic mortality at concentrations lower than the one shown by Meuer et al. (1989).

In conclusion, a decrease in albumen pH during early incubation due to an increased CO_2 concentration in the incubator may have increased the percentage of embryos that continued their development at the onset of incubation. However, the hypercapnic incubation at concentrations between 0.70% to 0.80% during the first 5 days of incubation was probably maintained too long causing the negative effect on embryonic development and hatchability.
Based on the results of the current thesis, it is not proven that the change in albumen pH is related to the percentage of embryos that continued their development at the onset of incubation. It is, therefore, unclear whether the micro-environment of the embryo during early incubation is involved in the negative effects of prolonged egg storage.

Meijerhof (1992) hypothesized that prestorage incubation or frequent warming during storage increases the metabolic activity of the embryo and, therefore, lowers the pH in the tissues by CO_2 production as a byproduct of metabolism. Chapter 5 showed that although the stages of embryonic development increased due to prestorage incubation and frequent warming during storage, the total albumen pH was not affected by these treatments at the end of storage

or during early incubation. However, it is possible that the CO_2 production of the embryo only affects the pH in the environment close to the embryo. The results of the increased CO_2 concentrations in the storage room (Chapter 3) suggest that the albumen pH during storage is not related to embryo survival. However, the results of the high CO_2 concentrations during early incubation suggest that the albumen pH during early incubation may be important for embryo survival (Chapter 5). However, in Chapter 5, no interactions were found between prestorage incubation and hypercapnic incubation or between frequent warming during storage and hypercapnic incubation for early embryonic mortality or hatchability. Thus, the differences in albumen pH close to the embryo due to differences in the stages of embryonic development were probably not present or were not related to embryo survival.

Effects of Prolonged Egg Storage on Chick Quality

Several authors have shown that prolonged egg storage has a negative effect on chick quality on the day of hatch and on subsequent performance (Merritt, 1964; Byng and Nash, 1962; Tona et al., 2003, 2004). In Chapter 4, it was also shown that prolonged egg storage had a negative effect on chick quality on the day of hatch in terms of a lower yolk-free body mass, a shorter chick length, and a higher percentage of second-grade chicks. Singal and Kosin (1969) suggested that prolonged egg storage may induce irreversible changes in metabolism, which lowers the capacity of cells to maintain vital activities during subsequent incubation. Singal and Kosin (1969) placed a graft of splenic tissue on the chorio allantois membrane of stored and non-stored embryos. They observed a reduction in mitosis and protein synthesis when eggs were stored for a prolonged time. In addition, Fasenko (1996) and Christensen et al. (2001) observed that embryos stored for a prolonged time relied more on gluconeogenesis for the growth and function of organs during pipping and hatching than short-term stored embryos. The reduction of mitosis and protein synthesis and/or the increased gluconeogenesis during the energy demanding hatching process may have caused the negative effects of prolonged egg storage on chick quality. Molenaar et al. (2009) showed that the incubation of eggs at a sub-optimal high eggshell temperature of 38.9°C instead of the optimal 37.8°C (Lourens et al., 2005) from day 7 of incubation until hatch decreased the yolk-free body mass, protein and energy content of the chick, and chick length. The reason for the reduced development may be caused by the shorter incubation duration and the difference in nutrient utilization. Embryos incubated at an eggshell temperature of 38.9°C were less efficient with their proteins that they utilize from the yolk than embryos incubated at the optimal eggshell temperature. The proteins that were lost, may be used for the energy demanding hatching process because the hepatic glycogen amount on day 18 of incubation was less in eggs that were incubated at an eggshell temperature of 38.9°C than in eggs incubated at the optimal eggshell temperature. While,

carbohydrates are needed during hatching because O_2 is restricted at that time. The results of Molenaar et al. (2009) showed that the nutrient use is different between incubation at a high and an optimal eggshell temperature and this may have caused differences in chick quality. The negative effects of prolonged egg storage on chick quality may be as well caused by differences in protein or carbohydrate metabolism as was indicated by Singal and Kosin (1969), Fasenko (1996), and Christensen et al. (2001).

Lourens et al. (2005) showed that eggs should be incubated at an optimal eggshell temperature of 37.8°C throughout incubation to optimize hatchability and chick quality. In studies conducted before 2005, incubation temperature was often maintained at one temperature throughout incubation. This single temperature would result in eggshell temperatures that were higher than the optimal 37.8°C during the last part of incubation due to the heat production of the embryos. When long-term stored embryos are incubated at eggshell temperatures above 37.8°C, the negative effects on chick quality may be enhanced because both the prolonged egg storage and the sub-optimal incubation temperatures may negatively affect the same metabolic processes.

In Chapters 3, 4, and 5, the eggshell temperature was maintained at the optimal temperature of 37.8°C (Lourens et al., 2005) throughout incubation by a continuous adjustment of the incubator temperature. By using this procedure, the negative effects of prolonged egg storage on chick quality was probably minimized.

In the current thesis, prestorage incubation, frequent warming during storage, storage air composition, preincubation warming profile, and hypercapnic incubation did not affect chick quality on the day of hatch in terms of yolk-free body mass, chick length, and the percentage of second-grade chicks. These results suggest that the treatments used in the current thesis did not affect the processes in the embryo that determine chick quality. Treatments, such as the in ovo feeding of carbohydrates (Uni et al., 2005), may be able to minimize the negative effects of prolonged egg storage on chick quality. However, we suggest that the incubation of eggs at an optimal eggshell temperature of 37.8°C (Lourens et al., 2005) throughout incubation also minimizes the negative effects of prolonged egg storage on chick quality.

Conclusions

From the current thesis, it can be concluded that prolonged egg storage decreases hatchability due to an increase in embryonic mortality during the first 9 days of incubation.

The differences in embryonic mortality from days 0 to 9 of incubation between batches of eggs are marginal when embryos are not developed beyond stage EG13. When embryos are developed beyond stage EG13, due to prestorage incubation, frequent warming during storage,

or high environmental temperatures, the embryonic mortality from days 0 to 9 of incubation can increase to more than 50%. At oviposition, the stage of embryonic development often ranges from EG10 to EG13, and these are the optimal stages to survive prolonged egg storage and to continue embryonic development at the onset of incubation successfully after prolonged egg storage. When prestorage incubation or frequent warming during storage is used to minimize the negative effects of prolonged egg storage, the risk for decreased hatchability is, therefore, higher than the chance for improvement.

It cannot be concluded from the current thesis whether the stage of embryonic development, the number of viable embryonic cells, or both determine the sensitivity of the embryo to prolonged egg storage, because both prestorage incubation and frequent warming during storage increased the stage of embryonic development and the number of viable embryonic cells.

The storage air compositions, studied in the current study, do not affect embryonic development, hatchability, or chick quality, when eggs are stored for 14 days at a storage temperature of 16°C and a relative humidity of 75%.

A preincubation warming profile of 24 hours decreases embryonic mortality during the first 9 days of incubation and, therefore, increases hatchability compared to a preincubation warming profile of 4 hours. Why the 24-h preincubation warming profile improves hatchability is unclear.

It is also unclear whether the change in albumen pH during early incubation is involved in the negative effects of prolonged egg storage. Hypercapnic incubation decreases the total albumen pH during early incubation, which may increase the number of embryos that continues their development at the onset of incubation. These results suggest that albumen pH during early incubation partially repairs the negative effects of prolonged egg storage. In contrast, hypercapnic incubation retards embryonic development and increases early and late embryonic mortality. A possible explanation for this negative effect is that the hypercapnic incubation at CO_2 concentrations between 0.70% to 0.80% during the first 5 days of incubation is too long.

Although some of the treatments used in the current thesis affect hatchability, none of the treatments affect chick quality on the day of hatch in terms of yolk-free body mass, chick length, or the percentage of second-grade chicks.

Because the treatments that affected embryonic development sometimes improved hatchability, it seems that embryo characteristics have a more important role in the negative effects of prolonged egg storage on hatchability than changes in the egg characteristics, such as changes in the albumen pH and albumen height.

References

Arora, K. L., and I. L. Kosin. 1966. Changes in the gross morphological appearance of chicken and turkey blastoderms during preincubation storage. Poult. Sci. 45:819-825.

Arora, K. L., and I. L. Kosin. 1967. Autoradiographic and cytological study of blastodermal cells in turkey eggs subjected to extended pre-incubation storage. Bio. Bull. 133:303-309.

Arora, K. L., and I. L. Kosin. 1968. The response of the early chicken embryo to pre-incubation temperature as evidenced from its gross morphology and mitotic pattern. Physiol. Zool. 41:104-112.

Asmundson, V. S., and J. J. MacIlraith. 1948. Preincubation tests with turkey eggs. Poult. Sci. 27:394-401.

Bakst, M. R., and V. Akuffo. 1999. Impact of egg storage on embryonic development. Avian Poult. Biol. Rev. 13:125-131.

Bakst, M. R., S. K. Gupta, and V. Akuffo. 1997. Comparative development of the turkey and chicken embryo from cleavage through hypoblast formation. Poult. Sci. 76:83-90.

Bakst, M. R., and L. Holm. 2003. Impact of egg storage on carbonic anhydrase activity during early embryogenesis in the turkey. Poult. Sci. 82:1193-1197.

Bakst, M. R., and B. Howarth Jr. 1977. Hydrolysis of the hen's perivitelline layer by cock sperm. Biol. Reprod. 17:370-379.

Becker, W. A. 1960. The storage of hatching eggs and the post-hatching body weights of chickens. Poult. Sci. 39:588-590.

Becker, W. A. 1964. The storage of white leghorn hatching eggs in plastic bags. Poult. Sci. 43:1109-1112.

Becker, W. A., and G. E. Bearse. 1958. Pre-incubation warming and hatchability of chicken eggs. Poult. Sci. 37:944-948.

Becker, W. A., J. V. Spencer, and J. L. Swartwood. 1963. Storing hatching eggs in plastic bags. Poult. Sci. 42:1256.

Becker, W. A., J. V. Spencer, and J. L. Swartwood. 1967. Hatchability of eggs held in plastic bags at two temperatures. Poult. Sci. 46:311-314.

Becker, W. A., J. V. Spencer, and J. L. Swartwood. 1968. Carbon dioxide during storage of chicken and turkey hatching eggs. Poult. Sci. 47:251-258.

Benton, C. E., and J. Brake. 1996. The effect of broiler breeder flock age and length of egg storage on egg albumen during early incubation. Poult. Sci. 75:1069-1075.

Bellairs, R. 1986. The primitive streak. Anat. Embryol. 174:1-14.

Berg, L. R. 1945. The relationship of clutch position and time interval between eggs to eggshell quality. Poult. Sci. 24:555-563.

Bloom, S. E., D. E. Muscarella, M. Y. Lee, and M. Rachlinski. 1998. Cell death in the avian blastoderm: resistance to stress-induced apoptosis and expression of anti-apoptotic genes. Cell Death Differ. 5:529-538.

Board, R. G., and R. Fuller. 1974. Non-specific antimicrobial defences of the avian egg, embryo and neonate. Biol. Rev. 49:15-49.

Bowling, J. A., and B. Howarth Jr. 1981. The effects of exposing broiler breeder eggs to high temperatures before storage on hatchability and subsequent performance of chicks. Poult. Sci. 60:2333-2336.

Brake, J., T. J. Walsh, and S. V. Vick. 1993. Hatchability of broiler eggs as influenced by storage and internal quality. Zootech. Int. 16:30-41.

Brillard, J. P. 1993. Sperm storage and transport following natural mating and artificial insemination. Poult. Sci. 72:923-928.

Brillard, J. P., and G. R. McDaniel. 1986. Influence of spermatozoa numbers and insemination frequency on fertility in dwarf broiler breeder hens. Poult. Sci. 65:2330-2334.

Bruggeman, V., A. Witters, L. De Smit, M. Debonne, N. Everaert, B. Kamers, O. M. Onagbesan, P. Degraeve, and E. Decuypere. 2007. Acid-base balance in chicken embryos (*Gallus domesticus*) incubated under high CO_2 concentrations during the first 10 days of incubation. Respir. Physiol. Neurobiol. 159:147-154.

Burley, R. W., and D. V. Vadehra. 1989. The avian egg, chemistry and biology. John Wiley and Sons, New York, USA.

Buttgereit, F., and M. D. Brand. 1995. A hierarchy of ATP-consuming processes in mammalian cells. Biochem. J. 312:163-167.

Byng, A. J., and D. Nash. 1962. The effect of egg storage on hatchability. Br. Poult. Sci. 3:81-87.

Christensen, V. L., M. J. Wineland, G. M. Fasenko, and W. E. Donaldson. 2001. Egg storage effects on plasma glucose and supply and demand tissue glucogen concentrations of broiler embryos. Poult. Sci. 80:1729-1735.

Coleman, J. W., and P. B. Siegel. 1966. Selection of body weight at eight weeks of age. 5. Embryonic state at oviposition and its relationship to hatchability. Poult. Sci. 45:1003-1011.

Cunningham, F. E. 1974. Changes in egg white during incubation of the fertile egg. Poult. Sci. 53:1561-1565.

De Smit, L., V. Bruggeman, M. Debonne, J. K. Tona, B. Kamers, N. Everaert, A. Witters, O. Onagbesan, L. Arckens, J. De Baerdemeaker, and E. Decuypere. 2008. The effect of nonventilation during early incubation on embryonic development of chicks of two commercial broiler strains differing in ascites susceptibility. Poult. Sci. 87:551-560.

De Smit, L., V. Bruggeman, J. K. Tona, M. Debonne, O. Onagbesan, L. Arckens, J. De Baerdemaeker, and E. Decuypere. 2006. Embryonic developmental plasticity of the chick: Increased CO_2 during early stages of incubation changes the developmental trajectories during prenatal and postnatal growth. Comp. Biochem. Physiol. 145:166-175.

De Wit, A. A. C., and Th. A. M. Kruip. 2001. Bovine cumulus-oocyte-complex-quality is reflected in sensitivity for α-amanitin, oocyte-diameter and developmental capacity. Anim. Reprod. Sci. 65:51-65.

Edwards, C. L. 1902. The physiological zero and the index of development for the eggs of the domestic fowl (*Gallus Domesticus)*. Am. J. Physiol. 6:351-396.

Elibol, O., S. D. Peak, and J. Brake. 2002. Effect of flock age, length of egg storage, and frequency of turning during storage on hatchability of broiler hatching eggs. Poult. Sci. 81:945-950.

Eyal-Giladi, H. 1984. The gradual establishment of cell commitments during the early stages of chick development. Cell Differ. 14:245-255.

Eyal-Giladi, H. 1991. The early embryonic development of the chick as an epigenetic process. Crit. Rev. Poult. Biol. 3:143-166.

Eyal-Giladi, H., and S. Kochav. 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. Dev. Biol. 49:321-337.

Eyal-Giladi, H., D. Raveh, N. Feinstein, and M. Friedländer. 1979. Glucogen metabolism in the prelaid chick embryo. J. Morph. 161:23-38.

Fasenko, G. M. 1996. Embryo and poult viability in stored eggs. PhD thesis, University of Alberta, Edmonton, Canada.

Fasenko, G. M., V. L. Christensen, M. J. Wineland, and J. N. Petite. 2001a. Examining the effects of prestorage incubation of turkey breeder eggs on embryonic development and hatchability of eggs stored for four or fourteen days. Poult. Sci. 80:132-138.

Fasenko, G. M., R. T. Hardin, and F. E. Robinson. 1992a. Relationship of hen age and egg sequence position with fertility, hatchability, viability, and preincubation embryonic development in broiler breeders. Poult. Sci. 71:1374-1383.

Fasenko, G. M., F. E. Robinson, J. G. Armstrong, J. S. Church, and R. T. Hardin. 1991. Variability in preincubation embryo development in domestic fowl. 1. Effects of nest holding time and method of egg storage. Poult. Sci. 70:1876-1881.

Fasenko, G. M., F. E. Robinson, and R. T. Hardin. 1992b. Research Note: variability in preincubation embryonic development in domestic fowl. 2. Effects of duration of egg storage period. Poult. Sci. 71:2129-2132.

Fasenko, G. M., F. E. Robinson, A. I. Whelan, K. M. Kremeniuk, and J. A. Walker. 2001b. Prestorage incubation of long-term stored broiler breeder eggs: 1. Effects on hatchability. Poult. Sci. 80:1406-1411.

Fasenko, G. M., J. L. Wilson, F. E. Robinson, and R. T. Hardin. 1999. Effects of length of egg nest holding time and high environmental temperatures on prestorage embryonic development, survival, and hatchability of broiler breeders. J. Appl. Poult. Res. 8:488-492.

Fromm, D. 1964. Strength distribution, weight and some histological aspects of the vitelline membrane of the hen's egg yolk. Poult. Sci. 43:1240-1246.

Fromm, D. 1966. The influence of ambient pH on moisture content and yolk index of the hen's yolk. Poult. Sci. 45:374-379.

Funk, E. M., and H. V. Biellier. 1944. The minimum temperature for embryonic development in the domestic fowl (*Gallus domesticus*). Poult. Sci. 23:538-540.

Funk, E. M., and J. Forward. 1960. Effect of holding temperature on hatchability of chicken eggs. Miss. Agric. Exp. Station Bull. 732:3-12.

Gildersleeve, R. P., and D. P. Boeschen. 1983. The effects of incubator carbon dioxide level on turkey hatchability. Poult. Sci. 62:779-784.

Gillespie, J. I., and S. McHanwell. 1987. Measurement of intra-embryo pH during early stages of development in the chick embryo. Cell Tissue Res. 247:445-451.

Glücksmann, A. 1951. Cell deaths in normal vertebrate ontogeny. Biol. Rev. 26:59-86.

Goodrum, J. W., W. M. Britton, and J. B. Dabis. 1989. Effect of storage conditions on albumen pH and subsequent hard-cooked egg peelability and albumen shear strength. Poult. Sci. 68:1226-1231.

Gordon, C. D., and H. S. Siegel. 1966. Storage of pedigreed hatching eggs in cryovac. Poult. Sci. 45:1369-1371.

Gupta, S. K., and M. R. Bakst. 1993. Turkey embryo staging from cleavage through hypoblast formation. J. Morph. 217:313-325.

Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morph. 88:49-92.

Hays, F. A., and C. Nicolaides. 1934. Variability in development of fresh-laid hen eggs. Poult. Sci. 13:74-90.

Hill, D. 2001. Chick Length uniformity profiles as a field measurement of chick quality? Avian Poult. Biol. Rev. 12:188.

Hodgetts, B. 1999. Incubation and hatching. Page 53 in The Poultry Production Guide. K. Naheeda, ed. Elsevier International, Doetichem, the Netherlands.

Horne, P. L. M. 2009. Productiekosten van kuikenvlees. Een internationale vergelijking (Production costs of poultry meat. An international comparison). Rapport 2009-004, LEI Wageningen UR, The Hague, the Netherlands.

Howarth Jr., B., and S. T. Digby. 1973. Evidence for the penetration of the vitelline membrane of the hen's ovum by a trypsin-like acrosomal enzyme. J. Reprod. Fertil. 33:123-125.

Hulet, R. M., V. L. Christensen, and L. G. Bagley. 1987. Controlled egg weight loss during incubation of turkey eggs. Poult. Sci. 66:428-432.

Hurnik, G. I., B. S. Reinhart, and J. F. Hurnik. 1978. Relationship between albumen quality and hatchability in fresh and stored hatching eggs. Poult. Sci. 57:854-857.

Jacobson, M. D., M. Weil, and M. C. Raff. 1997. Programmed cell death in animal development. Cell 88:347-354.

Jansonius, F. A. T., F. F. Putirulan, and R. S. Kaltofen. 1976. De delingsaktiviteit in het blastoderm van een aantal broedeieren, afkomstig van White Plymouth Rock hennen onmiddelijk na leg bepaald (Mitotic activity in blastoderms of White Plymouth Rock hens determined after oviposition). Intern rapport pluimveeonderzoek "Het Spelderholt", Beekbergen, the Netherlands.

Kaufman, L. 1938. Entwicklung und Wachstum von Hühnerembryonen in frischen und in gelagerten Eiern. Arch. Geflügelk. 13:63-77.

Kochav, S., M. Ginsburg, and H. Eyal-Giladi. 1980. From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. II. Microscopic anatomy and cell population dynamics. Dev. Biol. 79:296-308.

Konishi, T., and I. L. Kosin. 1974. Morbidity of aging non-incubated chicken blastoderms: further cytological evidence and interpretation. J. Embryol. Exp. Morph. 32:557-571.

Kosin, I. L. 1956. Studies on pre-incubation warming of chicken and turkey eggs. Poult. Sci. 35:1384-1392.

Kosin, I. L., and K. L. Arora. 1966. The pattern of early embryonic development in two genetically isolated lines of Broad Breasted Bronze turkeys. Poult. Sci. 45:622-629.

Kosin, I. L., and T. Konishi. 1973. Pre-incubation storage conditions and their effect on the subsequent livability of chicken embryos: exogenous CO_2, plastic bags and extended holding periods as factors. Poult. Sci. 52:296-302.

Krueger, W. F., J. H. Quisenberry, and J. W. Bradley. 1965. Preserving hatching power of eggs through the use of plastic bags. Poult. Sci. 44:1392.

Lapão, C., L. T. Gama, and M. Chaveiro Soares. 1999. Effects of broiler breeder age and length of egg storage on albumen characteristics and hatchability. Poult. Sci. 78:640-645.

Lockshin, R. A., and Z. Zakeri. 2004. Introduction. Pages 3-23 in When cells die II. R. A. Lockshin, and Z. Zakeri, ed. John Wiley & Sons, Hoboken, New Jersey, USA.

Lodge, J. R., N. S. Fechheimer, and R. G. Jaap. 1971. The relationship of *in vivo* sperm storage interval to fertility and embryonic survival in the chicken. Biol. Reprod. 5:252-257.

Lourens, A. 2006. Heating eggs before storage increases hatchability. World Poult. 22(4):22-23.

Lourens, A., H. van den Brand, M. J. W. Heetkamp, R. Meijerhof, and B. Kemp. 2007. Effects of eggshell temperature and oxygen concentration on embryo growth and metabolism during incubation. Poult. Sci. 86:2194-2199.

Lourens, A., H. van den Brand, R. Meijerhof, and B. Kemp. 2005. Effect of eggshell temperature during incubation on embryonic development, hatchability and posthatch development. Poult. Sci. 84:914-920.

Mather, C. M., and K. F. Laughlin. 1976. Storage of hatching eggs: the effect on total incubation period. Br. Poult. Sci. 17:471-479.

Mather, C. M., and K. F. Laughlin. 1979. Storage of hatching eggs: the interaction between parental age and early embryonic development. Br. Poult. Sci. 20:595-604.

Mayes, F. J., and M. A. Takeballi. 1984. Storage of the eggs of the fowl (*Gallus domesticus)* before incubation: a review. World's Poult. Sci. J. 40:131-140.

McKerley, R. G., G. W. Newell, J. G. Berry, G. V. Odell, and R. D. Morrison. 1967. The effects of some acidic and alkaline atmospheres on the changes in pH and haugh units in chicken eggs. Poult. Sci. 46:118-132.

McNally, E. H. 1943. Some characteristics of the ovomucin gel of egg white. Poult. Sci. 22:25-29.

Meijerhof, R. 1992. Pre-incubation holding of hatching eggs. World's Poult. Sci. J. 48:57-68.

Meijerhof, R. 1994. Theoretical and empirical studies on temperature and moisture loss of hatching eggs during the pre-incubation period. PhD thesis, Landbouwuniversiteit Wageningen, Wageningen, the Netherlands.

Meijerhof, R., J. P. T. M. Noordhuizen, and F. R. Leenstra. 1994. Influence of pre-incubation treatment on hatching results of broiler breeder eggs produced at 37 and 59 weeks of age. Br. Poult. Sci. 35:249-257.

Meijerhof, R., and B. van Beek. 1993. Mathematical modelling of temperature and moisture loss of hatching eggs. J. Theor. Biol. 165:27-41.

Meir, M., and A. Ar. 1987. Improving turkey poult quality by correcting incubator humidity to match eggshell conductance. Br. Poult. Sci. 28:337-342.

Meir, M., and A. Ar. 1998. Pre-incubation warming as a means of lengthening storage time of fertile eggs. Pages 825-829 in Proc. 10th European Poultry Conference, Israel. World's Poultry Science Association, Israel Branch, Jerusalem, Israel.

Merritt, E. S. 1964. Pre-incubation storage effects on subsequent performance of chickens. Br. Poult. Sci. 5:67-73.

Meuer, H. J., and R. Baumann. 1988. Oxygen pressure in intra- and extraembryonic blood vessels of early embryo. Resp. Physiol. 71:331-342.

Meuer, H. J., U. Sieger, and R. Baumann. 1989. Measurement of pH in blood vessels and interstitium of 4 and 6 days-old chick embryos. J. Dev. Physiol. 11:354-359.

Molenaar, R., R. Meijerhof, B. Kemp, R. Hulet, and H. van den Brand. 2009. High eggshell temperatures: A matter of life and death importance? Avian Biol. Res. 2:252-253.

Molenaar, R., I. A. M. Reijrink, R. Meijerhof, H. van den Brand. 2008. Relationship between hatchling length and weight on later productive performance in broilers. World's Poult. Sci. J. 64:599-603.

Nalbandov, A., and L. E. Card. 1943. Effect of stale sperm on fertility and hatchability of chicken eggs. Poult. Sci. 22:218-226.

Nechaeva, M. V., H. Töhardt, A. Hühnke, I. G. Makarenko, and T. M. Turpaev. 2004. Effects of environmental factors on the amnion rhythmic contractions in chick embryogenesis. Avian Poult. Biol. Rev. 15:137-144.

Olson, M. W., and R. M. Fraps. 1944. Maturation, fertilization and early cleavage of the egg of the domestic turkey. J. Morph. 74:297-309.

Onagbesan, O., V. Bruggeman, L. De Smit, M. Debonne, A. Witters, K. Tona, N. Everaert, and E. Decuypere. 2007. Gas exchange during storage and incubation of avian eggs: effects on embryogenesis, hatchability, chick quality and post-hatch growth. World's Poult. Sci. J. 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. Poult. Sci. 41:98-103.

Proudfoot, F. G. 1964a. The effects of plastic packaging and other storage treatments on hatching eggs. Poult. Sci. 43:87-95.

Proudfoot, F. G. 1964b. The effect of nitrogen and other gases on the hatchability of eggs stored in plastic bags. Can. J. Anim. Sci. 44:120-121.

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. Poult. Sci. 44:636-644.

Proudfoot, F. G. 1966. Hatchability of stored chicken eggs as affected by daily turning during storage and prewarming and vacuuming eggs enclosed in plastic with nitrogen. Can. J. Anim. Sci. 46:47-50.

Proudfoot, F. G. 1969. The handling and storage of hatching eggs. Pages 127-141 in The fertility and hatchability of the hen's egg. T. C. Carter, and B. M. Freeman, ed. Oliver and Boyd, Edinburgh, UK.

Proudfoot, F. G. 1972. Influence of an improved hatching-egg storage method on the subsequent performance of broiler chickens. Can. J. Anim. Sci. 52:303-308.

Proudfoot, F. G., and H. W. Hulan. 1982. Effect of preincubation warming on the hatchability of hens eggs from normal and semidwarf parental genotypes. Can. J. Anim. Sci. 62:321-322.

PVE (Productboard for livestock, meat, and eggs). 2008. Statistics livestock, meat, and eggs (Kengetallen vee, vlees en eieren). The Hague, the Netherlands.

Raddatz. E., H. Eyal-Giladi, and P. Kucera. 1987. Patterns of oxygen consumption during establishment of chephalocaudal polarity in the early chick embryo. J. Exp. Zool. Suppl. 1:213-218.

Raveh, D., M. Friedländer, and H. Eyal-Giladi. 1971. Organelle differentiation in the chick blastoderm during hypoblast formation. Wilhelm Roux' Arch. 166:287-299.

Raveh, D., M. Friedländer, and H. Eyal-Giladi. 1976. Nucleolar ontogenesis in the uterine chick germ correlated with morphogenetic events. Exp. Cell Res. 100:195-203.

Reijrink, I. A. M., R. Meijerhof, B. Kemp, E. A. M. Graat, and H. van den Brand. 2009. Influence of prestorage incubation on embryonic development, hatchability, and chick quality. Poult. Sci. 88:2649-2660.

Reijrink, I. A. M., R. Meijerhof, B. Kemp, and H. van den Brand. 2008. The chicken embryo and its micro environment during egg storage and early incubation. World's Poult. Sci. J. 64:581-598.

Reinhart, B. S., and G. I. Hurnik. 1982. Hatching performance of cryovac enclosed hatching eggs stored in a high humidity environment. Poult. Sci. 61:564-566.

Renema, R. A., J. J. R. Feddes, K. L. Schmid, M. A. Ford, and A. R. Kolk. 2006. Internal egg temperature in response to preincubation warming in broiler breeder and turkey eggs. J. Appl. Poult. Res. 15:1-8.

Robinson, F. E. 2002. Management for control of ovarian development in broiler breeders. Avian Med. 59:1-7.

Robinson, F. E., R. T. Hardin, N. A. Robinson, and B. J. Williams. 1991. The influence of egg sequence position on fertility, embryo viability and embryo weight in broiler breeders. Poult. Sci. 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. Poult. Sci. 34:754-760.

Sadler, W. W., H. S. Wilgus, and E. G. Buss. 1954. Incubation factors affecting hatchability of poultry eggs. 2. Some effects of carbon dioxide upon morphogenesis. Poult. Sci. 33:1108-1115.

SAS Institute Inc. 2004. SAS/STAT User's Guide. Version 9.1. SAS Inst. Inc., Cary, NC.

Scott, T. A., and F. G. Silversides. 2000. The effect of storage and strain of hen on egg quality. Poult. Sci. 79:1725-1729.

Scott, H. M., and D. C. Warren. 1936. Influence of ovulation rate on the tendency of the fowl to produce eggs in clutches. Poult. Sci. 15:381-389.

Shenstone, F. S. 1968. The gross composition, chemistry and physico-chemical basis of organization of the yolk and white. Pages 26-58 in Egg Quality: A study of the hen's egg. T. C. Carter, ed. Oliver and Boyd, Edinburgh, UK.

Silversides, F. G., and T. A. Scott. 2001. Effect of storage and layer age on quality of eggs from two lines of hens. Poult. Sci. 80:1240-1245.

Singal, D. P., and I. L. Kosin. 1969. Induced preincubation aging of the avian egg and subsequent development of the embryo, as revealed by the DNA, RNA, and protein level of its spleen. Proc. Soc. Exper. Biol. Med. 132:871-877.

Spratt, N. T., and H. Haas. 1960. Integrative mechanisms in development of the early chick blastoderm. I. Regulative potentiality of separated parts. J. Exp. Zool. 145:97-137.

Steinke, L. 1969. Weitere Keimscheibeuntersuchungen an unbebrüteten Hühnereiern. Arch. Geflügelk. 33:133-146.

Steinke, L. 1972. Keimscheibeuntersuchungen an Hühnereiern unter besonderer Berücksichtigung des Entwicklungszustandes. Arch. Geflügelk. 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. Pages 81-90 in Avian Incubation. G. Tullet, ed. Butterworth-Heinemann, London, UK.

Storey, K. B., and J. M. Storey. 2004. Metabolic rate depression in animals: transcriptional and translational controls. Biol. Rev. 79:207-233.

Stroikin, Y., H. Dalen, U. T. Brunk, and A. Terman. 2005. Testing the "garbage" accumulation theory of ageing: Mitotic activity protects cells from death induced by inhibition of autophagy. Biogerontology 6:39-47.

Taneja, G. C., and R. S. Gowe. 1962. Effect of varying doses of undiluted semen on fertility and hatchability in the domestic fowl. J. Reprod. Fertil. 4:161-174.

Taylor, L. W., and G. O. Kreutziger. 1965. The gaseous environment of the chick embryo in relation to its development and hatchability. 2. Effect of carbon dioxide and oxygen levels during the period of the fifth through the eight days of incubation. Poult. Sci. 44:98-106.

Taylor, L. W., R. A. Sjodin, and C. A. Gunns. 1956. The gaseous environment of the chick embryo in relation to its development and hatchability. 1. Effect of carbon dioxide and oxygen levels during the first four days of incubation upon hatchability. Poult. Sci. 35:1206-1215.

Terman, A., B. Gustafsson, and U. T. Brunk. 2006. Mitochondrial damage and intralysosomal degradation in cellular aging. Mol. Aspects Med. 27:471-482.

Tona, K., F. Bamelis, B. De Ketelaere, V. Bruggeman, V. M. B. Moreas, J. Buyse, O. Onagbesan, and E. Decuypere. 2003. Effects of egg storage time on spread of hatch, chick quality, and chick juvenile growth. Poult. Sci. 82:736-741.

Tona, K., O. Onagbesan, B. De Ketelaere, E. Decuypere, and V. Bruggeman. 2004. Effects of age of broiler breeders and egg storage on egg quality, hatchability, chick quality, chick weight, and posthatch growth to forty-two days. J. Appl. Poult. Res. 13:10-18.

Uni, Z., P. R. Ferket, E. Tako, and O. Kedar. 2005. In ovo feeding improves energy status of late-term chicken embryos. Poult. Sci. 84:764-770.

Van Schalkwyk, S. J., Z. Brand, S. W. P. Cloete, and C. R. Brown. 1999. Effect of time of egg collection and pre-incubation treatment on blastoderm development and embryonic mortality in ostrich embryos. S. Afr. J. Anim. Sci. 29 (3):154-163.

Verstegen, M. W. A., W. van der Hel, H. A. Brandsma, A. M. Henken, and A. M. Bransen. 1987. The Wageningen respiration unit for animal production research: A description of the equipment and its possibilities. Pages 21-48 in Energy Metabolism in Farm Animals: Effects of housing, stress and disease. M. W. A. Verstegen, and A. M. Henken, ed. Martinus Nijhoff Publishers, Dordrecht, the Netherlands.

Vick, S. V., J. Brake, and T. J. Walsh. 1993. Relationship of incubation humidity and flock age to hatchability of broiler hatching eggs. Poult. Sci. 72:251-258.

Walsh, T. J., R. E. Rizk, and J. Brake. 1995. Effects of temperature and carbon dioxide on albumen characteristics, weight loss, and early embryonic mortality of long stored hatching eggs. Poult. Sci. 74:1403-1410.

Warren, D. C., H. A. Roff, and E. Long. 1965. Hatchability of eggs stored in plastic-lined egg cases. Poult. Sci. 44:1278-1280.

Warren, D. C., and H. M. Scott. 1935a. The time factor in egg formation. Poult. Sci. 14:195-207.

Warren, D. C., and H. M. Scott. 1935b. Physiological factors influencing the rate of egg formation in the domestic hen. J. Agric. Res. 51:565-572.

Whittow, G. C. 1986. Regulation of body temperature. Pages 221-268 in Avian Physiology. P. D. Sturkie, Ed. Springer-Verlag, New York, USA.

Willemsen, H., N. Everaert, A. Witters, L. De Smit, M. Debonne, F. Verschuere, P. Garain, D. Berckmans, E. Decuypere, and V. Bruggeman. 2008. Critical assessment of chick quality measurements as an indicator of posthatch performance. Poult. Sci. 87:2358-2366.

Williams, K. C. 1992. Some factors affecting albumen quality with particular reference to haugh unit score. World's Poult. Sci. J. 48:5-16.

Wilson, H. R. 1991. Physiological requirements of the developing embryo: temperature and turning, Pages 145-156 in Avian Incubation. S. G. Tullet, ed. Butterworths, London, UK.

Witters, A. 2009. Factors affecting the impact of hypercapnia during early egg incubation and its effects on pre- and postnatal physiological parameters of broilers. PhD thesis, Katholieke Universiteit Leuven, Leuven, Belgium.

Wolanski, N. J., E. J. Luiten, R. Meijerhof, and A. L. J. Vereijken. 2004. Yolk utilisation and chick length as parameters for embryo development. Avian Poult. Biol. Rev. 15:233-234.

Yassin, H., A. G. J. Velthuis, M. Boerjan, J. van Riel, and R. B. M. Huirne. 2008. Field study on broiler eggs hatchability. Poult. Sci. 87:2408-2417.

Yassin, H., A. G. J. Velthuis, M. Boerjan, and J. van Riel. 2009. Field study on broilers' first-week mortality. Poult. Sci. 88:798-804.

Yoo, B. H., and E. Wientjes. 1991. Rate of decline in hatchability with preincubation storage of chicken eggs depends on genetic strain. Br. Poult. Sci. 32:733-740.

Summary

After oviposition, hatching eggs are first stored at the breeder farm, then transported to the hatchery and stored again before they are finally set in the incubator. The storage duration depends on the supply of hatching eggs, hatchery capacity, and market demand for day-old chicks. Normally, commercial hatcheries set their eggs after 3 to 5 days of storage to minimize negative effects of egg storage on hatchability and chick quality. However, hatcheries may need to increase the storage duration in some situations. It is well known that an increase in the storage duration increases incubation duration, decreases hatchability, decreases chick quality on the day of hatch, decreases subsequent growth performance, and increases posthatch mortality. Although the negative effects of prolonged egg storage are well known, it is not totally understood why this extended storage has negative effects on hatchability and chick quality.

During storage, eggs are stored below temperatures at which morphological development occurs. However, some cellular activity still occurs in the embryo, such as cell death. Besides changes in the embryo, also changes in the egg characteristics (micro-environment of the embryo) occur during storage. After oviposition, CO_2 and H_2O are lost from the egg and albumen pH increases from about 7.6 to about 9.0, yolk pH increases from about 6.0 to about 6.5, albumen height decreases, and strength of the yolk membrane decreases as well. Because the described changes in the embryo and egg characteristics both occur during egg storage, it is difficult to distinguish which of these changes are involved in the negative effects of prolonged egg storage on hatchability and chick quality.

To investigate if the changes in the embryo, in the egg characteristics (micro-environment of the embryo), or both are involved in the negative effects of prolonged egg storage, several authors either changed the stage of embryonic development by using prestorage incubation or changed the egg characteristics by using different storage air compositions. In these studies, hatchability was often the only factor that was determined and the effect on the embryo and egg characteristics were not measured. Furthermore, hatchability results were often in conflict with each other. These conflicting results may be explained by unmeasured differences in embryo viability caused by strain, breeder flock age, storage duration, or storage conditions.

The first aim of the current thesis was to investigate which physiological mechanisms are involved in the negative effects of prolonged egg storage on hatchability and chick quality. The second aim was to investigate how the negative effects of prolonged egg storage can be reduced by making changes in the storage or early incubation conditions. The results of the different experiments in the current thesis are presented in a chronological order. Previous research

that was described in a literature review (Chapter 1) suggested that the effect of prolonged egg storage on hatchability and chick quality depends on the stage of embryonic development, the total number of viable embryonic cells, and the micro-environment of the embryo during storage and/or early incubation. To investigate if the stage of embryonic development determines the sensitivity of an embryo to prolonged egg storage, prestorage incubation was used as a treatment. The effect of prestorage incubation on the stage of embryonic development, hatchability, and chick quality was investigated in two experiments (Chapter 2). In experiment I, eggs were collected from a Cobb broiler breeder flock of 61 weeks of age at 3, 5, 8, and 12 days before setting. In experiment II, eggs were collected from a Cobb broiler breeder flock of 28 weeks of age at 5 and 11 days before setting. After egg collection, half of the eggs were stored immediately at 16°C to 18°C, and the other half were exposed to prestorage incubation for 6 hours in experiment I and for 4.5 hours in experiment II. Prestorage incubation only affected hatchability when egg storage was beyond 8 days. In experiment I, prestorage incubation increased the stage of embryonic development from EG11.67 to EG13.26 and decreased hatchability by 6.2% when storage duration was 12 days. In experiment II, prestorage incubation increased the stage of embryonic development from EG9.22 to EG12.63 and improved hatchability by 5.3% when storage duration was 11 days. It seemed that prestorage incubation had a positive effect on hatchability when the majority of embryos were below developmental stage EG10 at egg collection. To understand more about the role of the stage of embryonic development in the negative effects of prolonged egg storage, data from Chapters 2 and 5 were combined with data from literature and a pilot study (Chapter 6). These combined data indicated that embryonic mortality during the first 9 days of incubation can increase above 50% when the majority of the embryos are developed beyond stage EG13 during egg storage. In addition, the combined data suggested that prestorage incubation or frequent warming during storage improved hatchability when the majority of the embryos are less developed than developmental stage EG10 at egg collection, which agreed with the conclusion in Chapter 2. The effect of prestorage incubation and frequent warming during storage on embryonic mortality from days 0 to 9 of incubation seems to be negligible when embryos are at the developmental stages EG10, EG11, EG12, and EG13 at egg collection and do not develop further than developmental stage EG13 due to prestorage incubation or frequent warming during storage.

In addition to the stage of embryonic development, the conditions in the micro-environment of the embryo during storage may also determine negative effects of prolonged egg storage on hatchability and chick quality. To change the micro-environment of the embryos, eggs were stored in normal air, 0.74% CO_2, 1.5% CO_2, or 3.0% O_2. The effect of these treatments on albumen quality, embryonic development, hatchability, and chick quality were investigated (Chapter 3). Eggs were collected from a Ross broiler breeder flock of 38 weeks of age at 14 days prior to setting. Storage temperature was 16°C, and relative humidity was 75%. Results

demonstrated that the 0.74% and 1.5% CO_2 treatments prevented part of the change in albumen height and albumen pH during storage, but the treatments did not affect embryonic development, hatchability, or chick quality. The low O_2 concentration in the storage air did not affect egg characteristics, embryonic development, hatchability, or chick quality.

To investigate whether the warming profile at the onset of incubation affects embryo viability in short- and long-term stored eggs, two experiments were conducted in which the effect of a 4-h and 24-h preincubation warming profile was investigated on embryonic development, hatchability, and chick quality (Chapter 4). In experiment I, three batches were used, and the age of the Ross broiler breeder flock increased from 41 weeks to 50 weeks. In experiment II, eggs were collected from a Ross broiler breeder flock at an age of 28 weeks. In both experiments, a 2 x 2 completely randomized design was used with: two storage durations (4 and 14 days at 17°C in experiment I and 4 and 13 days at 19°C in experiment II) and two preincubation warming profiles (within 4 or 24 hours from storage temperature to 37.8°C). Results demonstrated that in both experiments, the 24-h preincubation warming profile decreased embryonic mortality during the first 9 days of incubation by 4.4% and consequently increased hatchability when storage duration was 13 or 14 days. Due to the use of a low number of eggs in experiment I, these effects were only significant in experiment II. The preincubation warming profile did not affect hatchability when storage duration was 4 days and did not affect chick quality. In conclusion, embryonic mortality caused by prolonged egg storage can be decreased by a slow preincubation warming profile. The reason for the decline in embryonic mortality during the first 9 days of incubation is unclear.

In the experiment described in Chapter 5, eggs were exposed to prestorage incubation and frequent warming during storage to increase the stage of embryonic development and/or the number of viable embryonic cells. In addition, eggs were exposed to hypercapnic incubation during the first 5 days of incubation to alter albumen pH during early incubation. These three treatments were used to investigate the effect on egg characteristics, embryonic development, hatchability, and chick quality. It was also investigated whether the effect of hypercapnic incubation on hatchability depends on the stage of embryonic development at the onset of incubation. All eggs were stored for 15 days at 16°C and 75% relative humidity. On the second day after oviposition, half of the eggs were incubated for 7 hours (prestorage incubation). During storage, half of the eggs were warmed 6 times for 30 minutes in water at 37.8˚C (frequent warming during storage). During the first 5 days of incubation, the CO_2 concentration in the incubator was maintained between 0.70% and 0.80% (hypercapnic incubation) or increased from 0.05% to 0.20% (control). Results demonstrated that prestorage incubation and frequent warming during storage increased the stage of embryonic development and the number of viable embryonic cells. Consequently, incubation duration decreased, but hatchability and chick quality were not affected.

Hypercapnic incubation decreased total albumen pH and the percentage of eggs classified as infertile, which was 1.2% lower in the hypercapnic treatment than in the control treatment. In contrast, embryonic development was retarded and hatchability of fertile eggs was decreased by 1.3%. Chick quality was not affected. The effect of hypercapnic incubation on hatchability and chick quality did not depend on the stage of embryonic development at the onset of incubation.

From the current thesis, it can be concluded that prolonged egg storage decreases hatchability due to an increase in embryonic mortality during the first 9 days of incubation.

The differences in embryonic mortality from days 0 to 9 of incubation between batches of eggs are marginal when embryos are not developed beyond stage EG13. When embryos are developed beyond stage EG13, due to prestorage incubation, frequent warming during storage, or high environmental temperatures, the embryonic mortality from days 0 to 9 of incubation can increase to more than 50%. At oviposition, the stage of embryonic development often ranges from EG10 to EG13, and these are the optimal stages to survive prolonged egg storage and to continue embryonic development at the onset of incubation successfully after prolonged egg storage. When prestorage incubation or frequent warming during storage is used to minimize the negative effects of prolonged egg storage, the risk for decreased hatchability is, therefore, higher than the chance for improvement.

It cannot be concluded from the current thesis whether the stage of embryonic development, the number of viable embryonic cells, or both determine the sensitivity of the embryo to prolonged egg storage, because both prestorage incubation and frequent warming during storage increased the stage of embryonic development and the number of viable embryonic cells.

The storage air compositions, studied in the current study, do not affect embryonic development, hatchability, or chick quality, when eggs are stored for 14 days at a storage temperature of 16°C and a relative humidity of 75%.

A preincubation warming profile of 24 hours decreases embryonic mortality during the first 9 days of incubation and, therefore, increases hatchability compared to a preincubation warming profile of 4 hours. Why the 24-h preincubation warming profile improves hatchability is unclear.

It is also unclear whether the change in albumen pH during early incubation is involved in the negative effects of prolonged egg storage. Hypercapnic incubation decreases the total albumen pH during early incubation, which may increase the number of embryos that continues their development at the onset of incubation. These results suggest that albumen pH during early incubation partially repairs the negative effects of prolonged egg storage. In contrast,

hypercapnic incubation retards embryonic development and decreases hatchability. A possible explanation for this negative effect is that the hypercapnic incubation at CO_2 concentrations between 0.70% to 0.80% during the first 5 days of incubation is too long.

Although some of the treatments used in the current thesis affect hatchability, none of the treatments affect chick quality.

Because the treatments that affected embryonic development sometimes improved hatchability, it seems that embryo characteristics have a more important role in the negative effects of prolonged egg storage on hatchability than changes in the egg characteristics, such as changes in the albumen pH and albumen height.

Samenvatting

Nadat broedeieren gelegd zijn, worden ze eerst opgeslagen op het moederdierbedrijf. Na een paar dagen worden de eieren getransporteerd naar de broederij en worden ze nogmaals opgeslagen. Na een opslagperiode, die varieert in tijd, worden de eieren ingelegd in de broedmachine. De lengte van de opslagperiode hangt af van het aanbod van broedeieren, de capaciteit van de broederij en de vraag naar eendagskuikens. Om de negatieve effecten van de opslagperiode op het uitkomstpercentage en kuikenkwaliteit te minimaliseren is de opslagperiode meestal maximaal 3 tot 5 dagen. In sommige situaties worden eieren langer dan een week opgeslagen. Een verlenging van de opslagperiode verlengt het broedproces, vermindert het uitkomstpercentage, heeft een negatief effect op kuikenkwaliteit net na uitkomst en op de groei van de kuikens en verhoogt de sterfte van de kuikens na uitkomst. Ondanks dat de negatieve effecten van een lange opslagperiode algemeen bekend zijn, is het nog niet duidelijk waardoor de negatieve effecten van een lange opslagperiode worden veroorzaakt.

Tijdens de opslagperiode worden eieren opgeslagen onder een temperatuur waarbij de morfologische ontwikkeling van het embryo wordt stil gelegd. Desondanks zijn de cellen in het embryo actief. Er treedt onder andere celdood op. Naast deze veranderingen in het embryo, treden er tijdens de opslagperiode ook veranderingen op in de ei karakteristieken. Nadat het ei is gelegd, verliest het CO_2 en H_2O. Het albumen pH stijgt van 7.6 naar 9.0. De dooier pH stijgt van 6.0 naar 6.5. De hoogte van het albumen en de sterkte van het dooiermembraan nemen beide af. Omdat de beschreven veranderingen in het embryo en de ei karakteristieken beide optreden tijdens de opslagperiode, is het moeilijk te bepalen welke veranderingen betrokken zijn bij de negatieve effecten van een lange opslagperiode op het uitkomstpercentage en kuikenkwaliteit. Om te onderzoeken of de veranderingen in het embryo of in de ei karakteristieken betrokken zijn bij de negatieve effecten van een lange opslagperiode, hebben verschillende auteurs het ontwikkelingsstadium van het embryo verandert door eieren na verzamelen kort te broeden of hebben de veranderingen in de ei karakteristieken beïnvloedt door de gassamenstelling van de lucht in de opslagruimte te veranderen. In deze studies werd vaak alleen het effect op het uitkomstpercentage onderzocht. Het effect van de behandeling op de embryo- of ei karakteristieken werd meestal niet gemeten. Daarnaast waren de uitkomstpercentages tussen de verschillende studies vaak in conflict met elkaar. Deze tegenstrijdige resultaten kunnen mogelijk verklaard worden door niet gemeten verschillen in embryo kwaliteit veroorzaakt door ras, leeftijd van de moederdieren, lengte van de opslagperiode of opslagcondities.

Het eerste doel van dit onderzoek was om te onderzoeken welke fysiologische mechanismen betrokken zijn bij de negatieve effecten van een lange opslagperiode op het uitkomstpercentage en kuikenkwaliteit. Het tweede doel van dit onderzoek was om te onderzoeken hoe de negatieve effecten van een lange opslagperiode verminderd kunnen worden door de opslagcondities of vroege broedcondities te veranderen. De resultaten van de verschillende experimenten van dit proefschrift worden gepresenteerd in een chronologische volgorde. Eerder uitgevoerd onderzoek, dat werd beschreven in een literatuur overzicht (Hoofdstuk 1), suggereerde dat de effecten van een lange opslagperiode op het uitkomstpercentage en kuikenkwaliteit afhangt van het ontwikkelingsstadium van het embryo, het aantal goede embryo cellen en het microklimaat van het embryo tijdens opslag en/of vroege incubatie. Om te onderzoeken of de gevoeligheid van het embryo voor een lange opslagperiode bepaald wordt door het ontwikkelingsstadium van het embryo, werden eieren direct na het verzamelen voor een korte periode gebroed. In twee experimenten werd onderzocht of het kort broeden na het verzamelen effect had op het ontwikkelingsstadium van het embryo, het uitkomstpercentage en de kuikenkwaliteit (Hoofdstuk 2). In experiment I werden eieren verzameld van Cobb moederdieren van 61 weken oud, 3, 5, 8 en 12 dagen voor inleg in de broedmachine. In experiment II werden eieren verzameld van Cobb moederdieren van 28 weken oud, 5 en 11 dagen voor inleg in de broedmachine. Na het verzamelen van de eieren werd de helft van de eieren opgeslagen bij een temperatuur van 16 tot 18°C. De andere helft van de eieren werd in experiment I voor 6 uur gebroed en in experiment II voor 4.5 uur. Het kort broeden na het verzamelen had een effect op het uitkomstpercentage wanneer de opslagperiode langer was dan 8 dagen.

In experiment I nam het ontwikkelingsstadium toe van EG11.67 naar EG13.26 en nam het uitkomstpercentage af met 6.2% wanneer de eieren 12 dagen werden opgeslagen. In experiment II nam het ontwikkelingsstadium toe van EG9.22 naar EG12.63 en nam het uitkomstpercentage toe met 5.3% wanneer de eieren 11 dagen werden opgeslagen. Het leek erop dat het kort broeden van de eieren na het verzamelen een positief effect had op het uitkomstpercentage wanneer de meerderheid van de embryo's niet verder ontwikkeld waren dan stadium EG9 op het moment van verzamelen. Om meer te begrijpen over de rol van het ontwikkelingsstadium in de negatieve effecten van een lange opslagperiode werden gegevens van hoofdstuk 2 en 5 van dit proefschrift gecombineerd met gegevens uit de literatuur en gegevens van een pilot studie (Hoofdstuk 6). Deze gegevens gaven aan dat de embryonale sterfte tijdens de eerste 9 dagen van het broedproces kan toenemen tot meer dan 50% wanneer de meerderheid van de embryo's tijdens de opslagperiode verder ontwikkeld waren dan stadium EG13. De gecombineerde gegevens suggereerden ook dat het kort broeden van de eieren na het verzamelen of het frequent verwarmen van de eieren tijdens de opslagperiode het uitkomstpercentage verbeterde wanneer op moment van verzamelen de meerderheid van de embryo's niet verder ontwikkeld waren dan stadium EG9. Dit kwam overeen met de conclusie van hoofdstuk 2. Het effect van

het kort broeden van de eieren na het verzamelen of het frequent verwarmen van de eieren tijdens de opslagperiode op de embryonale sterfte in de eeste 9 dagen van het broedproces lijkt verwaarloosbaar wanneer de embryo's zich bevinden in de stadia EG10, EG11, EG12 en EG13 op moment van verzamelen en wanneer zij door het kort broeden na het verzamelen of tijdens de opslagperiode niet verder ontwikkelde dan EG13.

Naast het ontwikkelingsstadium van het embryo kunnen ook de condities in het microklimaat van het embryo een rol spelen in de negatieve effecten van de lange opslagperiode op het uitkomstpercentage en kuikenkwaliteit. Om het microklimaat van de embryo's te veranderen, werden eieren opgeslagen in normale lucht, 0.74% CO_2, 1.5% CO_2 of 3.0% O_2. Vervolgens werd onderzocht of deze behandelingen effect hadden op albumen kwaliteit, embryo ontwikkeling, het uitkomstpercentage en kuikenkwaliteit (Hoofdstuk 3). Eieren werden verzameld van een Ross moederdierkoppel van 38 weken oud, 14 dagen voor inleg in de broedmachine. De opslagtemperatuur was 16°C en de relatieve luchtvochtigheid was 75%. De 0.74% CO_2 en 1.5% CO_2 behandelingen voorkwamen een deel van de verandering in albumen hoogte en albumen pH die normaal optreedt tijdens de opslagperiode, maar de behandelingen hadden geen effect op embryonale ontwikkeling, het uitkomstpercentage of kuikenkwaliteit. Opslag bij een lage O_2 concentratie had geen effect op albumen kwaliteit, embryonale ontwikkeling, het uitkomstpercentage en kuikenkwaliteit.

Om te onderzoeken of het opwarmprofiel aan het begin van het broedproces invloed heeft op de kwaliteit van het embryo in kort en lang opgeslagen eieren werden 2 experimenten uitgevoerd (Hoofdstuk 4). In beide experimenten werd het effect van het opwarmprofiel op embryonale ontwikkeling, uitkomstpercentage en kuikenkwaliteit onderzocht. In experiment I werden 3 batches gebruikt en de leeftijd van de Ross moederdieren nam toe van 41 tot 50 weken. In experiment II werden eieren gebruikt van een Ross moederdierkoppel van 28 weken. In beide experimenten werd een 2 x 2 compleet gerandomiseerd ontwerp gebruikt met 2 opslagperiodes (4 en 14 dagen bij 17°C in experiment I en 4 en 13 dagen bij 19°C in experiment II) en 2 opwarmprofielen (in 4 of 24 uur van de opslagtemperatuur naar 37.8°C). Het opwarmprofiel van 24 uur verlaagde de embryonale sterfte in de eerste 9 dagen van het broedproces met 4.4% ten opzichte van het opwarmprofiel van 4 uur wanneer de eieren 13 of 14 dagen waren opgeslagen. Hierdoor verbeterde het uitkomstpercentage. Door het gebruik van te weinig eieren in experiment I waren deze positieve effecten alleen in experiment II significant. De twee opwarmprofielen hadden geen effect op het uitkomstpercentage wanneer de eieren 4 dagen waren opgeslagen en hadden ook geen effect op kuikenkwaliteit. Het kan geconcludeerd worden dat de embryonale sterfte die veroorzaakt wordt door een lange opslagperiode lager is wanneer eieren langzaam worden opgewarmd aan het begin van het broedproces. De reden voor de afname van de embryonale sterfte is niet bekend.

In het experiment dat bescheven is in Hoofdstuk 5 werd onderzocht of de gevoeligheid van een embryo voor een lange opslagperiode wordt bepaald door het ontwikkelingsstadium of door het aantal goede cellen. Tevens werd onderzocht of het albumen pH tijdens vroege incubatie betrokken is bij de negatieve effecten van een lange opslagperiode op het uitkomstpercentage en kuikenkwaliteit. Ook werd onderzocht of het effect van de veranderingen in het albumen pH tijdens vroege incubatie op het uitkomstpercentage afhankelijk is van het ontwikkelingsstadium van het embryo aan het begin van het broedproces. Alle eieren werden 15 dagen opgeslagen bij een temperatuur van 16°C en een relatieve luchtvochtigheid van 75%. Op de tweede opslagdag werd de helft van de eieren 7 uren gebroed. Tijdens de opslagperiode werd de helft van de eieren 6 keer 30 minuten opgewarmd in water van 37.8°C. Tijdens de eerste 5 dagen van het broedproces werd de helft van de eieren gebroed bij een CO_2 concentratie tussen de 0.70% en 0.80% om het albumen pH te verlagen, terwijl de andere helft van de eieren werd gebroed bij een oplopende CO_2 concentratie van 0.05% naar 0.20%. Er werd onderzocht of deze drie behandelingen effect hadden op ei karakteristieken, embryonale ontwikkeling, het uitkomstpercentage en kuikenkwaliteit.

Het broeden op de tweede opslagdag en het frequent opwarmen tijdens de opslagperiode verhoogde zowel het ontwikkelingsstadium van het embryo als het aantal goede embryo cellen. Hierdoor werd de duur van het broedproces verkort, maar het had geen effect op het uitkomstpercentage of kuikenkwaliteit. Het broeden bij een verhoogde CO_2 concentratie verlaagde de pH van het totale albumen en verminderde het percentage eieren dat werd geclassificeerd als onbevrucht met 1.2%. In tegenstelling tot deze resultaten, werd de embryonale ontwikkeling vertraagd en nam het uitkomstpercentage van bevruchte eieren af met 1.3%. Kuikenkwaliteit werd niet beïnvloed. Het effect van een verhoogde CO_2 concentratie tijdens de eerste 5 dagen van het broedproces op het uitkomstpercentage en kuikenkwaliteit werd niet beïnvloed door het ontwikkelingsstadium van het embryo aan het begin van het broedproces.

Aan de hand van de resultaten van dit proefschrift kan worden geconcludeerd dat een lange opslagperiode het uitkomstpercentage verlaagt door een toename van de embryonale sterfte tijdens de eerste 9 dagen van het broedproces.

Het verschil in embryonale sterfte in de eerste 9 dagen van het broedproces tussen verschillende groepen eieren is marginaal zolang embryo's niet verder ontwikkeld zijn dan stadium EG13. Wanneer embryo's verder ontwikkeld zijn dan stadium EG13 door eieren kort te broeden na het verzamelen, door eieren frequent te verwarmen tijdens de opslagperiode of door hoge omgevingstemperaturen, kan de embryonale sterfte tijdens de eerste 9 dagen van het broedproces toenemen tot boven de 50%. Direct na leg, varieert het ontwikkelingsstadium van het embryo vaak tussen EG10 en EG13. Deze stadia zijn het beste in staat om een lange opslagperiode te overleven en om de embryonale ontwikkeling succesvol te continueren na

een lange opslagperiode. Daarom is bij het gebruik van kort broeden na het verzamelen of het frequent opwarmen van eieren tijdens de opslagperiode het risico op een verslechtering van het uitkomstpercentage groter dan de kans op een verbetering van het uitkomstpercentage.

Aan de hand van de resultaten van dit proefschrift kan niet worden geconcludeerd of de gevoeligheid van het embryo voor een lange opslagperiode wordt bepaald door het ontwikkelingsstadium van het embryo of het aantal goede embryo cellen. Het kort broeden na het verzamelen en het frequent opwarmen van eieren tijdens de opslagperiode beïnvloedden namelijk beide zowel het ontwikkelingsstadium van het embryo als het aantal goede embryo cellen.

De gassamenstellingen van de lucht, die onderzocht zijn in dit proefschrift, hadden geen effect op embryonale ontwikkeling, het uitkomstpercentage of kuikenkwaliteit wanneer eieren 14 dagen werden opgeslagen bij 16°C en een relatieve luchtvochtigheid van 75%.

Een opwarmprofiel van 24 uur verminderde de embryonale sterfte tijdens de eerste 9 dagen van het broedproces en verbeterde daardoor het uitkomstpercentage ten opzichte van een opwarmprofiel van 4 uur. Waarom het opwarmprofiel van 24 uur het uitkomstpercentage verbeterde is niet bekend.

Het is ook niet duidelijk of de verandering in het albumen pH tijdens de eerste paar dagen van het broedproces betrokken is bij de negative effecten van een lange opslagperiode. Door de eerste 5 dagen van het broedproces te broeden bij een verhoogde CO_2 concentratie nam de pH van het totale albumen af. Deze afname heeft er mogelijk voor gezorgd dat meer embryos hun ontwikkeling continueerden aan het begin van het broedproces. Dit suggereert dat een verlaging van het albumen pH tijdens vroege incubatie een deel van de negatieve effecten van een lange opslagperiode verbetert. Darentegen, vertraagde de verhoogde CO_2 concentratie tijdens de eerste 5 dagen van het broedproces embryonale ontwikkeling en nam het uitkomstpercentage af. Een mogelijke verklaring voor dit negatieve effect is dat een CO_2 concentratie van 0.70% tot 0.80% te lang is vastgehouden.

Ondanks dat sommige behandelingen die uitgevoerd zijn in dit proefschrift een positief effect hadden op het uitkomstpercentage, werd kuikenkwaliteit niet beïnvloed.

Omdat de behandelingen die embryo ontwikkeling beïnvloedden soms een positief effect hadden op het uitkomstpercentage, lijkt het erop dat embryo karakteristieken een grotere rol spelen in de negatieve effecten van een lange opslagperiode op het uitkomstpercentage en kuikenkwaliteit dan veranderingen in de ei karakteristieken zoals veranderingen in albumen pH en albumen hoogte.

Curriculum Vitae

Curriculum Vitae (Nederlands)

Inge Reijrink werd geboren op 18 april 1981 in Tilburg en groeide op in Diessen. In 1999 behaalde zij haar VWO diploma aan de scholengemeenschap Koning Willem II College in Tilburg. Zij deed examen in de vakken Nederlands, Engels, Duits, Wiskunde, Scheikunde, Natuurkunde, Biologie en Economie I. In september van het jaar 1999 startte zij met de studie Zoötechniek aan Wageningen Universiteit & Research. Tijdens deze studie koos zij voor de specialisaties Gezondheidsleer en Reproductie en Agrarische Bedrijfseconomie. Voor de specialisatie Gezondheidsleer en Reproductie deed zij onderzoek naar de relatie tussen eikwaliteit en kuikenkwaliteit en de relatie van deze twee factoren met het immuunrespons tegen schapen rode bloedcellen. Voor de specialisatie Agrarische Bedrijfseconomie deed zij onderzoek naar de economische consequenties van strengere eisen omtrent the inputs voor de biologische melkveehouderij. Daarnaast liep zij stage bij het Institute of Veterinary, Animal and Biomedical Sciences at Massey University, Palmerston North, New Zealand en bij de Gezondheidsdienst voor Dieren (GD Varken) in Deventer. In het jaar 2004 is zij student assistent geweest bij de vakgroep Adaptatiefysiologie van Wageningen Universiteit & Research. In februari 2005 begon zij als junior onderzoekster op de onderzoeksafdeling van HatchTech Incubation Technology in Veenendaal. In oktober 2005 begon zij als promovenda bij de vakgroep Adaptatiefysiologie van Wageningen Universiteit & Research. Tevens bleef zij werken bij HatchTech Incubation Technology in Veenendaal. In september 2008 is zij met Paul van Roovert getrouwd. Na het afronden van het promotieonderzoek blijft zij werkzaam op de onderzoeksafdeling van HatchTech Incubation Technology in Veenendaal.

Curriculum Vitae (English)

Inge Reijrink was born on the 18th of April in the year 1981 in Tilburg. She spent her childhood in the village Diessen. In 1999, she graduated from high school Scholengemeenschap Koning Willem II College in Tilburg. The subjects at school were Dutch, English, German, Mathematics, Chemistry, Physics, Biology, and Economics. In September of the year 1999, she started with the study Animal Sciences at Wageningen University & Research. She specialized in Animal Health and Reproduction and in Agriculture Business Ecnonomics. For the specialization Animal Health and Reproduction she investigated the relationship between egg quality and chick quality and the relationship of these two factors with the immune response against sheep red blood cells. For the specialization Agriculture Business Economics she investigated the economic consequences of stricter rules concerning the inputs for the biological dairy farms. In addition,

she did her internship at the Institute of Veterinary, Animal and Biomedical Sciences at Massey University, Palmerston North, New Zealand and at the Animal Health Service (Department Pigs) in Deventer. In 2004, she worked as a student-assistant at the department Adaptation Physiology at Wageningen University & Research. In February 2005, she started as a junior scientist at the Research Department of HatchTech Incubation Technology in Veenendaal. In October 2005, she started her PhD at the department of Adaptation Physiology at Wageningen University & Research. She also continued her activities at HatchTech Incubation Technology in Veenendaal. In September 2008, she married to Paul van Roovert. After graduation, she continues her activities at the Research Department of HatchTech Incubation Technology in Veenendaal.

Publications

Refereed Scientific Journals

Hoskin, S. O., W. R. Pomroy, I. A. M. Reijrink, P. R. Wilson, and T. N. Barry. 2003. Effect of withholding anthelmintic treatment on autumn growth and internal parasitism of weaner deer grazing perennial ryegrass-based pasture or chicory. Proc. N. Z. Soc. Anim. Prod. 63:269-273.

Van den Brand, H., I. A. M. Reijrink, L. A. Hoekstra, and B. Kemp. 2008. Storage of eggs in water affects internal egg quality, embryonic development, and hatchling quality. Poult. Sci. 87:2350-2357.

Reijrink, I. A. M., R. Meijerhof, B. Kemp, and H. van den Brand. 2008. The chicken embryo and its micro environment during egg storage and early incubation. World's Poult. Sci. J. 64:581-598.

Molenaar, R., I. A. M. Reijrink, R. Meijerhof, H. van den Brand. 2008. Relationship between hatchling length and weight on later productive performance in broilers. World's Poult. Sci. J. 64:599-603.

Reijrink, I. A. M., R. Meijerhof, B. Kemp, E. A. M. Graat, and H. van den Brand. 2009. Influence of prestorage incubation on embryonic development, hatchability, and chick quality. Poult. Sci. 88:2649-2660.

Reijrink, I. A. M., D. Berghmans, R. Meijerhof, B. Kemp, and H. van den Brand. 2010. Influence of egg storage time and preincubation warming profile on embryonic development, hatchability, and chick quality. Poult. Sci. 89:1225-1238.

Conference Proceedings and Abstracts

Molenaar, R., I. A. M. Reijrink, R. Meijerhof, and H. van den Brand. 2007. Correlation between chick length and chick weight at hatch and slaughter weight and breast yield in broilers. Book of Abstracts of the 29th Poultry Science Symposium Biology of Breeding Poultry, 23 – 25 July 2007, Edinburgh, UK. pp. 40.

Reijrink, I. A. M., H. W. M. van Straaten, R. Meijerhof, B. Kemp, and H. van den Brand. 2008. Measurements of changes in the number of cells of the chicken blastoderm during egg storage. Book of Abstracts of the 23rd World's Poultry Congress, 30 June – 4 July 2008, Brisbane, Queensland, Australia. pp. 288.

Reijrink, I. A. M., D. Berghmans, R. Meijerhof, B. Kemp, and H. van den Brand. 2009. Effect of pre-warming profile on hatchability and chick quality. Book of Abstracts of International Poultry Scientific Forum, 26 – 27 January 2009, Atlanta, Georgia, USA. pp. 23.

Reijrink, I. A. M., L. A. G. van Duijvendijk, R. Meijerhof, B. Kemp, and H. van den Brand. 2009. Gas concentrations during storage do not affect hatchability and chick quality. Book of Abstracts of the 4th Workshop on Fundamental Physiology and Perinatal Development in Poultry, 10 – 12 September 2009, Bratislava, Slovakia. pp. 41.

Other Publications

Reijrink, I. A. M. Storage of the avain embryo. International Hatchery Practice 21, number 4, 2007. pp. 7-8

Reijrink, I. A. M. Good egg storage to obtain more chicks. World Poultry 25, 13 July 2009. pp. 24-25.

Training and Supervision Plan of the Graduate School WIAS

Name: Inge Reijrink
Group: Adaptation Physiology
Daily supervisor: Dr. ir. H. van den Brand
Supervisors: Dr. ing. R. Meijerhof and Prof. dr. ir. B. Kemp

	Year	ECTS
The Basic Package		
WIAS introduction course	2006	1,5
WIAS course Philosophy of Science and Ethics	2007	1,5
Scientific Exposure		
International Conferences		
Poultry Focus Asia, Bangkok, Thailand	2006	0,9
XII European Poultry Conference, Verona, Italy	2006	1,2
Poultry Science Symposium Biology of Breeding Poultry Congress, Edinburgh, UK	2007	0,9
XXIII World's Poultry Congress, Brisbane, Australia	2008	1,2
International Poultry Scientific Forum, Atlanta, USA	2009	0,6
Seminars and Workshops		
The 2nd combined Workshop of Fundamental Physiology of the European Working Group of Physiology and Perinatal Development in Poultry, Berlin, Germany	2005	0,6
WIAS Science Day, Wageningen, the Netherlands	2006, 2007, 2008, 2010	1,2
GROW Science Day, Maastricht, the Netherlands	2006	0,3
The 4th Workshop on Fundamental Physiology and Perinatal Development in Poultry, Bratislava, Slovakia	2009	0,6
Presentations		
Poster presentation at GROW Science Day, Maastricht, the Netherlands	2006	1,0
Oral presentation at WIAS Science Day, Wageningen, the Netherlands	2007	1,0
Poster presentation at Poultry Science Symposium Biology of Breeding Poultry Congress, Edinburgh, UK	2007	1,0
Oral presentation at XXIII World's Poultry Congress, Brisbane, Australia	2008	1,0
Oral presentation at International Poultry Scientific Forum, Atlanta, USA	2009	1,0
Oral presentation at the 4th Workshop on Fundamental Physiology and Perinatal Development in Poultry, Bratislava, Slovakia	2009	1,0
In-Depth Studies		
Disciplinary and Interdisciplinary Courses		
Training in early embryonic development, Maastricht University, Maastricht, the Netherlands	2006-2007	4,0
Training in early embryonic development, University of Alberta, Edmonton, Canada	2008	0,6
Epigenesis and Epigenetics	2008	1,0
Mathematical Modelling in Biology	2008	2,0
Advanced Statistics Courses		
Design of Animal Experiments	2007	1,0
Statistics for the Life Sciences	2007	1,5
PhD student's discussion group		
Poultry Discussion Group	2007-2008	0,3
MSc level courses		
Training in Incubation, Pennstate University, State College, USA	2006	0,9
Professional Skills Support Courses		
PhD Competence Assessment	2005	0,3
Course Supervising MSc Thesis Work	2006	0,6
Project and Time Management	2006	0,9
Course Personal Efficacy	2006	0,5
Techniques for Writing and Presenting a Scientific Paper	2007	1,2
Scientific Publishing	2009	0,3
Didactic Skills Training		
Lecturing		
Lecture Adaptatiefysiologie I	2009	0,3
Supervising MSc Theses		
Florian van der Hoeven (major thesis)	2006	2,0
Debbie Berghmans (major thesis)	2007	2,0
Linda Baarslag (major thesis)	2008	2,0
Gilian van Duijvendijk (major thesis)	2008	2,0
Total		**39,9**

Colophon

The research described in the current thesis was funded by HatchTech Incubation Technology B.V., Veenendaal, the Netherlands.

Eggs used in the current thesis were donated by Lagerwey Hatchery, Lunteren, the Netherlands.
This thesis is printed by Ponsen & Looijen B.V., Ede, the Netherlands.