ROUTE TO RESILIENCE:
LONG TERM EFFECTS OF INCUBATION TEMPERATURE AND EARLY FEEDING IN BROILERS

JAN H.J. WIJNEN

Propositions

1. Effects of early feeding on disease resilience should not be studied without considering the incubation phase.
(this thesis)

2. Fast growing broilers are resilient animals.
(this thesis)

3. Insects are the primary food source of the future.

4. Only male animals should be grown for meat production.

5. Paternity decreases the risk of mental health issues of PhD students.

6. Pandemics are good for science.

Propositions belonging to the thesis entitled

'Route to resilience:
Long term effects of incubation temperature and early feeding in broilers.'

Jan H. J. Wijnen
Wageningen, 1 July 2021

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

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A. P. J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 1 July 2022
at 1.30 p. m. in the Omnia Auditorium.

Jan Hendrikus Johannes Wijnen
Route to resilience: Long term effects of incubation temperature and early feeding in broilers
208 pages.
PhD Thesis, Wageningen University, Wageningen, the Netherlands (2022)
With references, with summary in English
ISBN: 978-94-6447-194-6
DOI: 10.18174/568137

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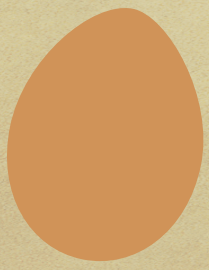

CHAPTER 1

GENERAL INTRODUCTION

Early life experiences can have long-lasting effects. A classic example of this comes from the so called 'Dutch hunger winter' that occurred during the second world war. Severe malnutrition of pregnant women during this winter increased the risk of obesity, schizophrenia, and high blood pressure during adulthood of their offspring (Ravelli et al., 1976; Susser et al., 1996; Stein et al., 2006). It has also been shown in many other animal species that early life experiences, particularly stress and under-nutrition, can affect health in later life (Rutherford et al., 2012). In broilers, effects of early life experiences on health in later life have barely been studied so far.

Almost 40% of the total global meat production comes from poultry (OECD/FAO, 2021). Therefore, broiler production undoubtedly has a considerable impact in the world in terms of food supply and usage of feed resources. Optimal broiler health contributes to sustainable production in terms of broiler welfare, ecological footprint, and human health. After all, suboptimal health increases the risk of diseases that can cause animal suffering, losses in production, inefficient conversion of feed resources, emergence of zoonoses, and increased usage of antibiotics, which in turn increases the threat of antimicrobial resistance. Given the possibility that early life experiences may affect broiler health in later life, conditions during early life should be optimized. Despite this, there are indications that some current nutritional and environmental conditions during the early life of broilers can perhaps be optimized. Therefore, this thesis presents a study on the effects of early life conditions on broiler health and, more specifically, broiler resilience.

DEFINITION OF RESILIENCE

The term 'resilience' is a catch-all term that is used in multiple disciplines. Despite its broad usage, the definition basically comes down to the same meaning; *the ability to cope with disturbances and reorganize with minimal loss of function* (Folke et al., 2010; Colditz and Hine, 2016). Loss of function can be determined in livestock by observing specific biomarkers, for instance, retardation of body growth or morbidity. Previous studies on livestock diseases indicate that resilience is mainly determined by three complementary aspects called resistance, tolerance, and recovery (Schneider and Ayres, 2008; Bishop, 2012). Resistance can be defined as the capability to withstand a disturbance; tolerance as the capability to keep negative impact to a minimum, despite being affected by a disturbance; and recovery as the capability to return to a normal state. To determine the total functional loss, broiler disease resilience was assessed in this thesis by studying these three aspects of resilience (Figure 1). A resilient broiler will have a higher resistance (longer timespan from exposure to a disturbance until first loss of function), a higher tolerance (milder functional loss, e.g. drop in growth rate), and/or faster recovery from a disturbance.

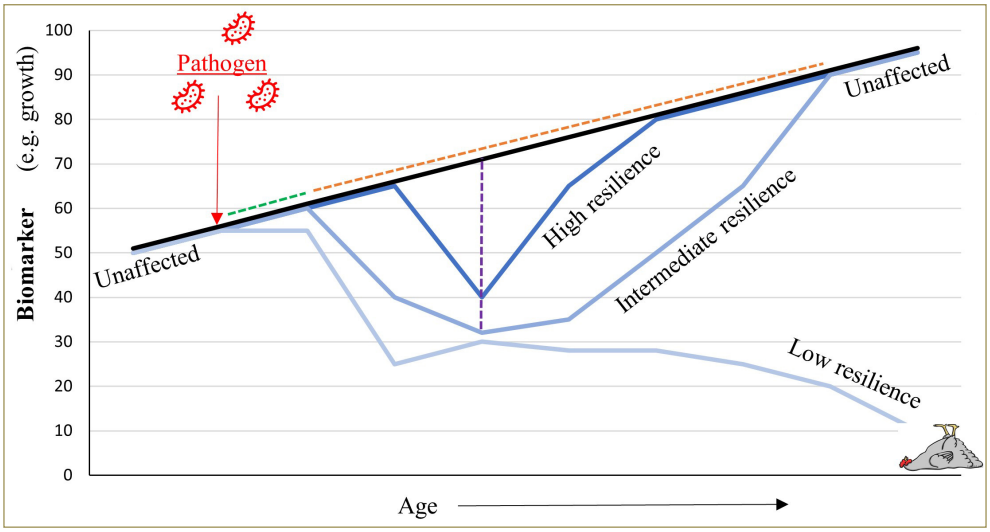

Figure 1. Graphical representation of 4 resilience levels (unaffected, high, intermediate, low) and the 3 complementary aspects that could be studied to measure resilience: resistance (green), tolerance (purple), and recovery (orange) from the intermediate resilience level.

It should be noted that some studies on disease resilience of livestock also include the capability to transmit infection to conspecifics as an additional aspect of resilience (Doeschl-Wilson et al., 2021). Transmissibility is determined at group level and could be studied by using an epidemiological approach. The current thesis does not follow such an approach.

BROILER DISEASE RESILIENCE

Throughout this thesis 'disease resilience' refers to resilience against pathogenic infections. Broiler diseases can have different causes, such as nutritional deficiencies or hereditable defects, but they can often be prevented either by breeding or by adjustments in diet formulations. Infectious diseases are difficult to prevent, and broilers can face a wide variety of diseases throughout their lifespan. Some occur seasonally, such as avian influenza, whereas others are ubiquitous in broiler houses, such as coccidiosis. Although the true prevalence of broiler infectious diseases is difficult to determine, it is evident that infectious diseases are a considerable threat to broiler production (Shirley et al., 2004; Zhang et al., 2013; Zhuang et al., 2014; Jones et al., 2019).

Various measures are taken to reduce the risk of infectious diseases. Large scale facilities often apply strict sanitary protocols and biosecurity measures (Berrang et al., 2000; Buhr et al., 2013; MacDonald and Wang, 2011). Although these protective measures largely reduce the pathogenic load in the environment, they do not offer full protection as infectious

diseases still occur. Additionally, drugs, such as anticoccidials, are used in diets, along with vaccinations or preventive usage of antibiotics. However, the extensive use of drugs over the past decades has contributed to antibiotic resistance. Consequently, antimicrobial and anticoccidial resistance now occurs frequently in poultry production all over the world (Peek and Landman, 2011; Ojimelukwe et al., 2018; Zhang et al., 2019; Veldman et al., 2021). Drug-resistant pathogens are a threat to poultry health as well as human health, because resistant bacteria that are found in poultry can be transferred to humans and can cause infections that are difficult or impossible to treat (reviewed by Picot et al., 2021). The World Health Organization stated that antimicrobial resistance is currently one of the most urgent public health threats. Some EU countries have banned preventive group treatments with antibiotics in livestock. In the Netherlands, such a ban resulted in an almost 70% reduction of antibiotic use and a simultaneous decrease in resistant isolates from livestock species (Veldman et al., 2021). New EU legislation (EU 2019/6) prohibited the routine use of antibiotics in farming from the 28th of January 2022 onwards.

This increasing threat of rising antimicrobial resistance and the ban on antibiotics, in addition to insufficient biosecurity measures, have drawn attention to alternative approaches to reduce the risk of infectious diseases in broilers. An alternative approach is to enhance broiler resilience. In this approach, the broiler's capacity to adapt to varying environments and stressors is increased by optimal development during early life. This is in contrast to the aforementioned measures that adapt the system to the broiler, because, as well as reducing drugs usage, it has the primary benefit of increasing broiler resilience in a non-pathogen specific manner. Higher broiler resilience is desirable because pathogens can mutate over time, requiring an ongoing development of new drugs, whereas enhancing broiler resilience supports general protection and may reduce the impact of antibiotic-resistant pathogens.

PERINATAL CONDITIONS

Broiler resilience may be enhanced by optimizing early life conditions. The early life of animals is generally recognized as a vulnerable phase of life, because some of their physiological regulatory systems are still immature. For example, broilers are not fully homeothermic at hatch and therefore depend on the ambient temperature to control their own body temperature (Whittow and Tazawa, 1991; Nichelmann and Tzschentke, 2002). The broiler's adaptive immune system is also immature at hatch and becomes functional during the first weeks of life (Shira et al., 2003). The maturation of these and other physiological regulatory systems does not seem to be a fixed process and early-life conditions, or so-called 'perinatal conditions', can affect these processes and permanently shape an animal's phenotype (Lindström, 1999; Dixon et al., 2016). The early life period can thus be seen as a window of opportunity in which perinatal conditions may alter broiler resilience in later life.

Post-hatch feeding strategy

One perinatal condition that may affect broiler resilience is the moment of first access to feed and water after hatch. In modern commercial practice, most chicks are withheld from feed and water during the period from hatch at a hatchery until placement in a broiler house (from here onwards referred to as '**delayed feeding**').

Delayed feeding

The duration from hatch until placement is dependent on hatch moment, chick processing procedures, and transport duration from hatchery to farm. Hatch moment varies between individual chicks within a batch of incubated eggs. The hatch moment is determined by abiotic factors, such as egg storage duration or temperature within an incubator, as well as biotic factors, such as variations in egg weight, parent flock age, sex of the embryo, and eggshell conductance (Meijer and Siemers, 1993; Tona et al., 2003;Almeida et al., 2008; Bergoug et al., 2013). The timespan between the first chick to hatch and the last within a batch is called the 'hatch window'. In commercial hatcheries, the average duration of a hatch window is around 33 hours (Olsen and Winton, 1941; Tona et al., 2003; Careghi et al., 2005). Several hours after the last chick hatches, all chicks are pulled from the incubator. This pull moment is set at a fixed duration of incubation at approximately 21 days and 8 hours (512 hours of incubation).

After pull, different chick processing procedures can take place such as quality assessment, vaccination, counting, and storage until transportation. Finally, chicks are transported from the hatchery to a farm. The duration of transport can vary tremendously between batches of chicks, depending on for instance the location of the farm or whether chicks have to be transported to multiple farm locations. Given these points, the duration of delayed feeding is estimated to be at least 9 h for late hatchers and 42 h for early hatchers in case of short transport durations, but longer durations of up to 72 h have been suggested (van de Ven et al., 2009; Willemsen et al., 2010).

After incubation, approximately 18% of the initial egg yolk still remains (Nangsuay et al., 2011), and this residual yolk (**RY**) is located in the abdomen of the newborn chick. This RY can be taken up by the chick via blood vessels or via a yolk stalk directly into the gastrointestinal tract up to 5 days post hatch (Esteban et al., 1991; Jeurissen et al., 1991; Noy et al., 1996; Noy and Sklan, 1998; van der Wagt et al., 2020). Therefore, a delay in feeding may not be such a problem for the neonatal chick. In fact, in the past it was common notion that direct access to feed and water after hatch would hamper the uptake of RY and that this would cause digestive problems, but once it was investigated this belief was dispelled (Roberts, 1928).

Early feeding

Since the beginning of the 21st century, alternative hatching systems have been introduced on the market in which chicks have access to feed and water directly after hatch (from here onwards referred to as '**early feeding**'). Many other studies on the effects of post-hatch feeding strategy in chickens followed since then (reviewed by Zubair et al., 1996; Noy and Sklan et al., 1997; Friedman et al., 2003; Noy and Uni, 2010, Willemsen et al., 2010; de Jong et al., 2017). In general, the perspective on early feeding gradually changed from negative to positive. It was consistently found that delayed fed broilers lose body weight during the fasting period whereas early fed broilers increase in body weight during the same period (Xin and Lee, 1997; Bigot et al., 2003; Careghi et al., 2005; van de Ven et al., 2009). In 2016, a meta-analysis was performed on 75 papers that studied the effects of post-hatch feeding strategy in broilers, layers, or turkey poults, with regard to growth performance (e.g., body weight, food conversion), physiology (e.g., hormone levels, organ weights, yolk size), behaviour, and health characteristics (e.g. morbidity, immunological parameters) (de Jong et al., 2017). The main conclusions were that early compared to delayed feeding temporarily increased plasma glucose and thyroid hormone concentrations, gut morphology, and relative weight of organs such as the liver, pancreas, and heart. In the long term, early compared to delayed feeding (24 - 84 hours) resulted in higher body weight (up to 6 weeks of age) and lower mortality rates. Feed conversion ratio was not affected by post-hatch feeding strategies.

Post-hatch feeding strategy vs disease resilience

More importantly in the context of this thesis, it was concluded that the effects of post-hatch feeding strategy on chicken health and welfare remain inconclusive (de Jong et al., 2017). There are some indications that post-hatch feeding strategy may affect broiler resilience. For example, de Jong et al. (2017) found that early feeding lowered total mortality during the first 6 weeks of age compared to delayed feeding. Mortality can be the result of pathogenic infection, and as such it could indicate an alteration in disease resilience. Also, post-hatch feeding strategy has been found to alter antibody and cytokine responses when exposed to model antigens such as anti-hemocyanin, human serum albumin, or sheep red blood cells (Shira et al., 2005; Simon et al., 2015; Lamot et al., 2016). This indicates that early fed broilers may differ in immune responses compared to delayed fed broilers and perhaps this improves their disease resilience. However, despite these findings, solid conclusions cannot be drawn on the effect of post-hatch feeding strategy on broiler resilience. For example, high or low antibody levels may indicate a difference in the strength of the immune response but they may also be the result of a higher or lower number of pathogens, respectively, that were able to infect the animal. Measuring disease responses to disease models with infectious pathogens may elucidate whether or not early feeding enhances broiler resilience.

Until now, four studies have investigated the effects of post-hatch feeding strategy on disease response in either layer or broiler chickens; all of them used coccidiosis and each study

found indications that early feeding may enhance disease resilience (Dibner et al., 1998; Yi et al., 2005; Walstra et al., 2010; Ao et al., 2012). However, those studies measured pathogenesis at few time points after induction of coccidiosis and therefore disease resilience could not be assessed as was previously defined in this thesis, by means of resistance, tolerance, and recovery to determine the total functional loss. Besides, in some of these studies treatment was confounded with other perinatal conditions. Furthermore, effects from those studies could not be attributed to early feeding as referred to in this thesis, because those early feeding treatments either had no water or had first access at placement in the broiler house. As previously described, due to the hatch window, some chicks are already over 42 hours old at placement in the broiler house. To avoid this, some studies decided to collect chicks from the incubator that hatched very recently (indicated by non-dried down feathers), but in such case the collected chicks all hatched at one specific time point within the hatch window, whilst it has been shown that early, midterm, and late hatching chicks may differ in their response to post-hatch feeding strategies (Sklan et al., 2000; Careghi et al., 2005; van de Ven et al., 2011; van de Ven et al., 2013; Lamot et al., 2014; Wang et al., 2014).

Incubation temperature

De Jong et al. (2017) demonstrated that considerable variation and inconsistency exists among studies on post-hatch feeding strategy, which may be dependent, in part, on incubation temperature. Incubation temperature has a major impact on embryo development. Avian embryos act poikilothermic during the majority of the incubation period (Dietz and van Kampen, 1994; French et al., 1997). Within certain limits, any increase in incubation temperature will raise embryo body temperature and accelerate metabolism, whereas the opposite will occur when lowering incubation temperature. Consequently, incubation temperature impacts the rate of embryo development and chick quality characteristics at hatch moment (Romanoff, 1936; Decuypere and Michels, 1992; Lourens et al., 2005).

Incubation temperature x post-hatch feeding strategy

It can be speculated that chicks that differ in development at hatch may respond differently to post-hatch feeding strategy. For instance, chicks that differ in yolk-free body mass (**YFBM**) or amount of RY at hatch may have different nutritional needs. Chicks with higher YFBM at hatch may need more energy and nutrients for maintenance and further development and thus their need for early feeding may be higher. Another difference that could arise is that of gut development through incubation temperature, which might impact response to first exogenous feed intake. It has been shown that incubation temperature can affect gut morphology and digestive enzyme activity in newborn chicks (Wineland et al., 2006; Barri et al., 2011; Leandro et al., 2017). Chicks with suboptimal gut development at hatch in terms of villi and crypt morphology or low digestive enzyme activity may have difficulties to digest and

absorb exogenous feed directly after hatch. These hypothesized biological mechanisms are yet to be tested and thus warrant further investigation.

Eggshell temperature

Traditionally, incubator air temperature was used to indicate the temperature at which eggs were incubated, however, actual egg temperature often differs from incubator air temperature (Booth, 1985; Tazawa and Rahn, 1987; Meijerhof and van Beek, 1993; Lourens et al., 2001; Piestun et al., 2009; Gualhanone et al., 2012; Tong et al., 2016). The difference between incubator air temperature and egg temperature varies between incubator types, depending, for instance, on sensor positions, uniformity within an incubator, and especially on airflow and cooling capacity during the last week of incubation (Kaltofen, 1969; van Brecht et al., 2005; Elibol and Brake, 2008; Özcan et al., 2010). Such discrepancies between air temperature and egg temperature hinder interpretability of studies on incubation air temperature. Alternatively, actual eggshell temperature (**EST**) can be measured as an accurate reflection of embryo body temperature (Spiers and Baummer, 1990; French, 1997). This thesis will merely reflect on studies that provided EST unless it is explicitly stated that incubation temperature was reflected by air temperature.

Effects of EST patterns on chick quality characteristics at hatch

In the 1920s, approximately 60 years after the introduction of the first artificial incubators, the first studies on incubation air temperatures were published (e.g. Voss, 1928). Almost 80 years later, the first studies investigating the effects of EST patterns were published and since then, a constant EST of 37.8°C throughout incubation is considered to result in the most optimal chick quality at hatch (Lourens et al., 2005). Later studies on EST showed that a relatively small increase to 38.6°C EST or a decrease to 36.7°C EST throughout the entire incubation period resulted in lower hatchability and worse chick quality at hatch expressed by worse navel conditions, higher RY weight and lower heart, stomach, and liver weights relative to yolk-free body mass (**YFBM**) (van der Pol et al., 2014).

Embryonic heat production starts from approximately day 7 of incubation and increases a 14 – 20 fold during the second and third week of incubation (Romijn and Lokhorst, 1960; Lourens et al., 2007; Nangsuay et al., 2013). With capacities of up to 200,000 eggs in commercial incubators, it can be difficult to remove excess heat during these weeks of incubation; consequently, higher EST than the optimal of 37.8°C can be found during the second and third weeks of incubation. To study the consequences of this, multiple studies have increased EST above 37.8°C either from mid incubation onwards (Lourens et al., 2007; Molenaar et al., 2010a; Molenaar et al., 2011a / 2011b; Molenaar et al., 2013; Ipek and Sozcu, 2014; Ipek et al., 2015; Zhu et al., 2015; Ipek and Sozcu, 2016; Nangsuay et al., 2016; Nangsuay et al., 2017) or only during the last week or days of incubation (Lourens et al., 2005; Joseph et al., 2006; Hulet et al., 2007; Leksrisompong et al., 2007; Oviedo-Rondon et al., 2009; Molenaar

et al., 2010; Barri et al., 2011; Maatjens et al., 2014a / 2014b; Sozcu et al., 2015; Almeida et al., 2016; Maatjens et al., 2016a / 2016b; Morita et al., 2016a / 2016b; Maatjens et al., 2017). The general conclusion from these studies is that, compared to a constant EST of 37.8°C, an EST of 38.9°C or higher from mid incubation onwards or only during late incubation lowers hatchability and impairs chick quality at hatch expressed by shorter chick length, lower YFBM, higher RY weight, worse navel condition, and lower relative organ weights. The lower chick quality was likely caused by a shorter incubation duration and an imbalance between oxygen availability and metabolic rate. A higher EST consistently decreases the incubation duration and consequently embryos have less time to develop within the egg (Molenaar et al., 2010a). As discussed, embryos act poikilothermic and therefore higher EST likely resulted in a higher metabolic rate and oxygen demand and initial faster development. However, from approximately embryonic day (**E**) 14 of incubation onwards, a plateau stage in oxygen availability occurs as maximal conductance of oxygen through the eggshell is reached (Visschedijk et al., 1985; Lourens et al., 2007; Nangsuay, 2016). In the situation of a high metabolic rate and limited availability of oxygen, the deposition of proteins from yolk into embryo body mass seems to be less efficient (Molenaar et al., 2010a; Molenaar et al., 2013). Proteins were probably used to supply energy, either via gluconeogenesis or via direct adenosine triphosphate production (Hazelwood and Lorenz, 1959; McArdle et al., 1981), whereas at a lower metabolic rate and sufficient oxygen availability, energy can be supplied by oxidation of fatty acids and proteins remain available for embryo body development (Moran, 2007; de Oliveira et al., 2008).

Alternative EST patterns

Although an EST of 37.8°C throughout incubation is generally considered optimal, recent findings indicate that two adjustments to this constant EST of 37.8°C may benefit embryo development. Firstly, it can be speculated that raising EST during the second week of incubation only may enhance chick development at hatch (Nangsuay, 2016). The availability of oxygen is not limited yet during this week and any EST higher than 37.8°C will raise embryo metabolism, potentially advancing body development. Nangsuay et al. (2017) showed that a higher EST of 38.9°C from E7 onwards resulted in higher YFBM at E14 and E16 compared to a constant EST of 37.8°C. In their study, the higher EST was maintained during the last week of incubation. Consequently, at E18 these differences disappeared and at hatch a lower YFBM was found for chicks incubated at 38.9°C EST from E7 onwards. Once oxygen availability becomes a limiting factor for embryo growth at approximately E14 onwards, it could be beneficial to lower EST again to 37.8°C to prevent the oxygen imbalance that was described in the previous paragraph. To the best of the author's knowledge, no studies have been conducted that have investigated the effects of raising EST continuously during the second week of incubation only on chick quality at hatch compared to a constant EST of 37.8°C throughout incubation.

The second finding is that lowering EST below 37.8°C during late incubation may be beneficial for embryo development and chick quality at hatch. As previously indicated, there is a plateau stage in oxygen availability during the last week of incubation (Visschedijk et al., 1985; Lourens et al., 2007; Nangsuay, 2016). An EST higher than 37.8°C during late incubation seems to result in a higher imbalance between metabolic rate and oxygen availability, resulting in impaired chick development at hatch (Molenaar et al., 2010b). Perhaps an EST slightly lower than 37.8°C during late incubation lowers this oxygen imbalance such that embryo development and chick quality at hatch are enhanced. Maatjens et al. (2016a) found that lowering EST to 36.7°C from E15, E17, or E19 of incubation onwards resulted in higher YFBM and higher relative weight of the liver and heart compared to a constant EST of 37.8°C (Maatjens et al., 2016a). It was also found that chicks at hatch had higher intestinal and bursal weights relative to YFBM when EST was lowered to 36.7°C from E19 of incubation onwards compared to a constant EST of 37.8°C (Maatjens et al., 2014a).

A lower EST of 36.7°C during late incubation may further enhance the suggested beneficial effects of a higher EST of 38.9°C during the second week of incubation (Figure 2). It was suggested by Nangsuay (2017) that a higher EST during mid incubation might result in an earlier and more severe shortage of oxygen during the remaining incubation period. A lower EST of 36.7°C during this remaining period may diminish this limitation, but this interaction is not investigated yet.

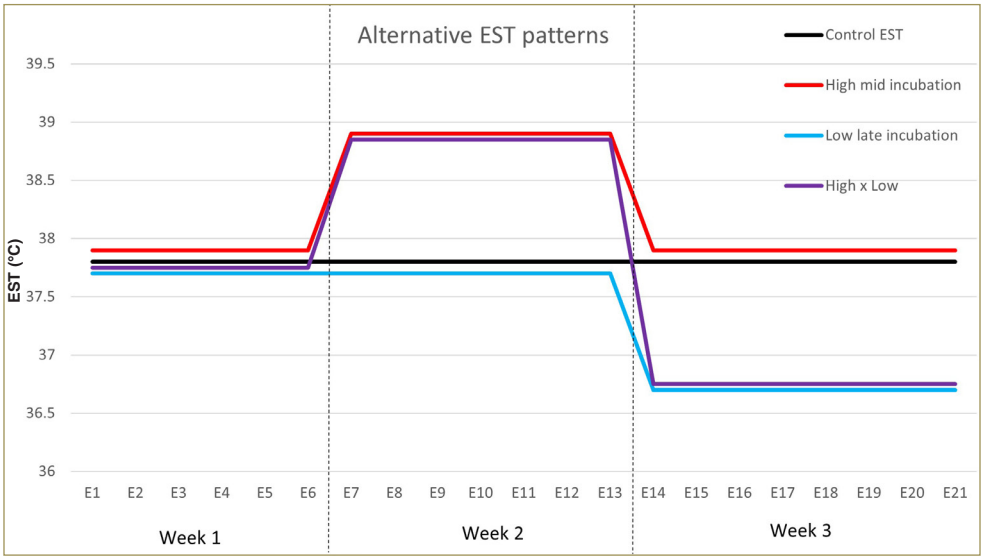

Figure 2. Graphical representation of alterations in EST pattern that may enhance embryo development.

Incubation temperature vs disease resilience

The incubation phase covers approximately 30% of a broiler's total lifespan. It is has been shown that EST patterns can affect broiler growth performance up to slaughter age (Joseph et al., 2006; Hulet et al., 2007). Therefore, it can be speculated that incubation temperature may also affect broiler resilience. Only two studies investigated effects of incubation temperature on immunocompetence of broilers during later life. One study showed that an air temperature over 38.8°C from E10 onwards compared to a constant air temperature of 37.8°C resulted in reduced colonization of *Salmonella Enteritidis* in the caecal content of broilers at 10 days post-hatch when inoculated with this bacterium at day 2 post-hatch (De Barros Moreira Filho et al., 2015). The other study with broilers found no altered immune responses when changing incubator air temperature by 1°C in either direction from 37.8°C during E14 and onwards, expressed by similar antibody levels at day 14 or 35 post-hatch when vaccinated at day 7 and 20 against a combination of Newcastle disease and infectious bursal disease (Santin et al., 2003).

In summary, studies investigating effects of incubation temperature on immunocompetence of broilers during later life are very limited, inconclusive, use incubator air temperature instead of EST, and did not study broiler resilience as defined in this thesis. Finally, no studies investigated the interaction with post-hatch feeding strategy.

AIM AND APPROACH OF THIS THESIS

In this thesis, the effects of post-hatch feeding strategy, EST pattern, and the interaction between the two, on broiler resilience to infectious diseases during rearing is studied.

The aim is to verify the following hypotheses;

1) Direct access to feed and water post-hatch results in a higher broiler disease resilience compared to delayed access.

2) A higher EST of 38.9°C during mid incubation in combination with a lower EST of 36.7°C during late incubation will result in optimal chick quality at hatch and a higher broiler disease resilience compared to a constant EST of 37.8°C.

3) Optimal incubation temperature and early feeding will interact synergistically to enhance broiler resilience.

To answer these hypotheses, three experiments were conducted (summarized in Figure 3). During the first experiment (Chapter 2 and 3), all chicks received early feed, so feeding strategy was not a treatment in this experiment. The effects of a higher EST during the second week of incubation, and a lower EST during the last week of incubation, and the interaction between them, on chick quality characteristics at hatch and growth performance until slaughter age were studied (Chapter 2). The effects of these EST patterns on bursa and jejunum morphology at hatch were also studied, as well as immune response during late life (Chapter 3).

Based on the findings of this experiment, it was decided that there was no further need to study the interaction between a higher EST during mid incubation and a lower EST during late incubation and these EST treatments were tested separately in the following two experiments. Furthermore, results from the first experiment were ambiguous on whether or not a lower EST of 36.7°C during the last week of incubation was advantageous for embryogenesis and immunocompetence during rearing. Therefore it was decided to lower EST at a slightly later moment during incubation in a second experiment, from embryonic day 17 instead of day 14 (each referred to as 'lower EST during late incubation' from here onwards).

In the second experiment of this thesis, the effects of a lower EST during late incubation and post-hatch feeding strategy and the interaction between them, on physiological regulatory systems such as thermoregulation and stress, during early life (Chapter 4) and broiler resilience (Chapter 5), were studied. To model broiler resilience, necrotic enteritis was induced at 3 weeks of age in collaboration with Poulpharm (Zwevegem, Belgium), a company that is specialized in clinical trials.

In the final experiment of this thesis, the effects of a higher EST during mid-incubation and post-hatch feeding strategy and the interaction between them on broiler resilience to colibacillosis was studied (Chapter 6). Colibacillosis was induced at one week of age according to a validated model from assistant professor and poultry veterinarian Dr. M. G. R. Matthijs (Utrecht University).

Disease morbidity, immune responses, and body weight losses after necrotic enteritis and colibacillosis were measured to study the three aspects of resilience described previously (resistance, tolerance, recovery). Necrotic enteritis and colibacillosis were chosen because they are currently common intestinal and respiratory diseases in the broiler industry. Each of the two diseases was induced at different ages post-hatch such that conclusions could be drawn on general broiler resilience. Moreover, both an intestinal and a respiratory disease were chosen to gain a broader insight into broiler resilience to various pathogens.

Findings from the three experiments are integrated and discussed in (Chapter 7), whereafter conclusions and practical implications as well as opportunities for future studies follow. Ultimately, insights from this thesis will result in optimization of perinatal conditions in such a way that broiler resilience will be improved, subsequently enhancing broiler health and welfare, improving financial gain, and reducing the need for antibiotics.

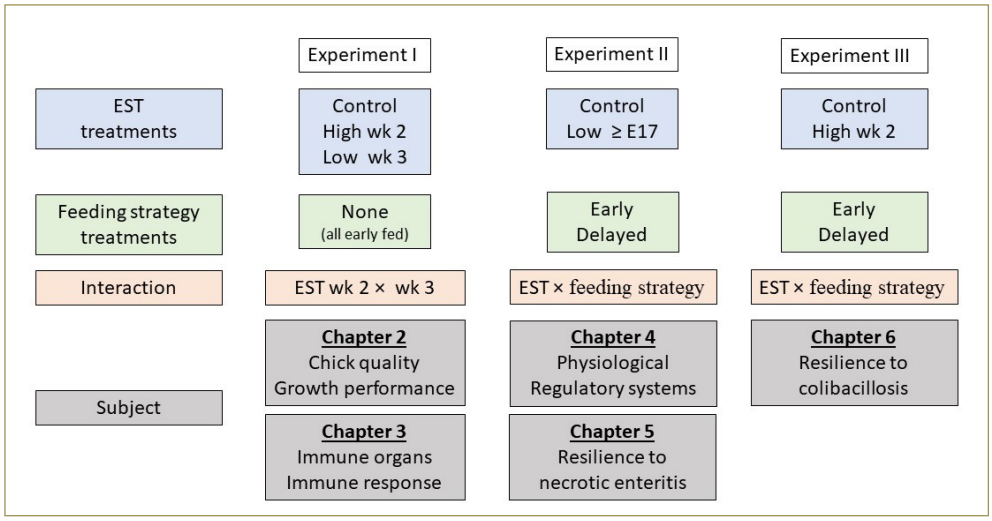

Figure 3. Graphical summary of the experiments that were conducted in this thesis including eggshell temperature (**EST**) and post-hatch feeding strategy treatments and themes per chapter of this thesis.

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CHAPTER 2

EFFECTS OF INCUBATION TEMPERATURE PATTERN ON BROILER PERFORMANCE

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Poultry Science (2020) 99, 3897 – 3907

ABSTRACT

During incubation, development of embryos is affected by eggshell temperature (**EST**). A constant EST of 37.8°C has been considered so far to result in most optimal embryo development. However, it can be hypothesized that a higher EST in wk 2 in combination with a lower EST in wk 3 stimulates embryo development and subsequent grow out performance. In this study, 468 eggs of a 44 wk old Ross 308 breeder flock were incubated at different incubation temperature patterns in a 2 x 2 factorial arrangement. In wk 2, EST was either 37.8°C or 38.9°C and in wk 3, EST was either 37.8°C or 36.7°C. At hatch, chick quality was determined. Thereafter, 320 broilers were grown in 32 pens (8 replicates / treatment) for 6 wk. Weekly BW and ADFI were determined and at d 40, slaughter yield from 128 broilers (4 per pen) was determined. Results showed that EST in wk 2 did not interact with EST in wk 3 for any variable. An EST of 38.9°C in wk 2 resulted in a 1 mm longer chick length (P<0.001) and 0.4 millimol/L lower blood glucose level (P=0.04) at hatch than an EST of 37.8°C. Grow out performance was not affected by EST in wk 2 of incubation. An EST of 36.7°C in wk 3 resulted in a 1 mm shorter chick length (P=0.02), 1.0 millimol/L higher blood glucose level (P<0.001), and higher relative heart (P=0.01) and stomach weights (P=0.03) at hatch than an EST of 37.8°C. Additionally, an EST of 36.7°C in wk 3 resulted in lower BW, ADG, and ADFI upon slaughter age (all P<0.03) than an EST of 37.8°C. In conclusion, no interaction between EST in wk 2 and 3 of incubation was found for any variable. A higher EST in wk 2 had minor effects at hatching and during rearing, whereas a lower EST in wk 3 seemed to result in better organ development, but resulted in lower grow out performance.

INTRODUCTION

Neonatal chick quality has been found to be related to later life performance (Fasenko and O'Dea, 2008; van de Ven et al., 2012). One of the factors affecting embryonic development and consequently neonatal chick quality is incubation temperature. Until now, a constant eggshell temperature (**EST**, reflecting embryo temperature) of 37.8°C throughout incubation is considered to result in the best embryo development and neonatal chick quality (Lourens et al., 2005).

However, Nangsuay (2016) speculated that a higher EST than 37.8°C during the second week of incubation might be beneficial for embryo development. It was shown that embryos had a higher yolk free body mass (**YFBM**) at embryonic day (**E**)14 and E16 when EST was raised to 38.9°C from E7 onwards compared to maintaining EST at 37.8°C continuously. When this higher EST of 38.9°C was maintained after E16 until hatch, differences in YFBM disappeared at E18 and at hatch YFBM was lower for chicks incubated at a higher EST compared to the control EST of 37.8°C. When temperature is raised during incubation, embryo metabolism increases as broiler embryos act mainly as poikilotherms (French, 1997), suggesting that embryonic development will be stimulated with a higher EST than 37.8°C. For the second week of incubation this might be true, but in the third week of incubation a higher EST than 37.8°C has been shown to negatively affect neonatal chick quality and post hatch performance (Romanoff, 1936; Lourens et al., 2005; Hulet et al., 2007; Leksrisompong et al., 2007; Ipek et al., 2014; Maatjens et al., 2014b, 2016a;). Hatchlings incubated at an EST higher than 37.8°C during late incubation showed a poorer quality, expressed by a shorter length, more residual yolk (**RY**), worse navel score, and lower weights of various organs relative to their YFBM. Moreover, a lower growth performance upon slaughter age has been found.

The cause of this impaired embryo development and neonatal chick quality might be related to an imbalance between embryonic metabolic rate and oxygen availability in the last part of incubation (Lourens et al., 2007). It has been shown that in the second week of incubation, oxygen availability is not limited (Lourens et al., 2007), but at approximately E14 embryo metabolic rate reaches its maximum increment (Nangsuay, 2016). With a higher temperature and consequently higher metabolic rate, more oxygen is needed. However, after E14, the amount of oxygen available to the embryo is limited by the conductance of the eggshell and consequently an imbalance between metabolic rate and oxygen availability seems to occur. To balance oxygen requirement and oxygen availability in wk 3 of incubation, it can be suggested that after E14 a lower EST than 37.8°C might be more optimal. Maatjens et al. (2016a, 2016b) demonstrated that an EST of 36.7°C or even 35.6°C from E15 onward resulted in a higher YFBM and higher relative heart, intestine, liver, and bursa of Fabricius weights of neonatal chicks than an EST of 37.8°C throughout incubation.

Based on the studies of Nangsuay et al. (2016) and Maatjens et al. (2016a, 2016b), it can be hypothesized that a combination of a higher EST in wk 2 and a lower EST in wk 3

of incubation may stimulate embryo development and chick quality at hatch compared to a constant EST of 37.8°C throughout incubation. Studies investigating these different EST patterns and the effects on post hatch performance are lacking. Therefore, the aim of this study was to investigate effects of different EST patterns during incubation on neonatal chick quality and growth performance of broilers.

MATERIAL AND METHODS

Experimental Design

The experiment was set up as a 2 x 2 factorial arrangement. All eggs (n=117 / treatment group) were incubated at an EST of 37.8°C until E7. From E7 until E15, eggs were incubated at an EST of either 37.8°C or an EST of 38.9°C. From E15 until hatch, eggs were incubated at an EST of either 37.8°C or an EST of 36.7°C. The experimental protocol was approved by the Governmental Commission on Animal Experiments, the Hague, the Netherlands; approval number: 2016.W-0087.001.

Incubation

Eggs of a 44 wk old Ross 308 broiler breeder flock were stored for 2 d at a storage temperature of 20°C at a commercial hatchery (Lagerwey BV, Lunteren, the Netherlands). Ten egg trays with 150 eggs each were bulk-weighed to determine the average egg weight from this batch of eggs. Thereafter, eggs were weighed individually and 468 first grade eggs within 3 g of the average egg weight were selected and divided into three weight classes: 62.0 – 62.9 g, 63.0 – 63.9 g, 64.0 – 64.9 g (156 eggs / weight class). Eggs were transported for approximately 30 min to the animal research facility of Wageningen University (Wageningen, the Netherlands), where eggs of each weight class were equally divided over the four treatment groups to exclude potential effects of initial egg weight on chick quality variables (e.g. body weight).

Before the start of incubation, all eggs were placed in one incubator. Eggs were warmed linearly in 14 h from storage temperature (20°C) to an EST of 37.8°C. The moment the eggs reached an EST of 37.8°C was considered to be the start of incubation (E0) and this EST was maintained until E7. The EST was monitored by 4 EST sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK), which were placed at the equator of the eggshell of 4 individual eggs, using silicone heat sink compound (Type 340; Dow Corning, Michigan, USA) and a small piece of duct-tape (approx. 1.5 x 1.5 cm). Incubator air temperature was continuously adjusted, based on the median temperature of the 4 EST sensors to maintain an EST of 37.8°C.

At E7, all eggs were candled and infertile eggs were removed. Thereafter, eggs of each weight class were equally divided over 4 incubators (2 replicates / treatment group). Each incubator had 4 EST sensors, which were attached to four individual eggs as described above. EST in each incubator was determined continuously and incubator air temperature

was adjusted automatically when necessary based on the median of the 4 EST sensors, as described above. From E7 until E15, two incubators were maintained at an EST of 37.8°C, whereas the other two incubators were maintained at an EST of 38.9°C.

At E15, all eggs were candled again and eggs containing a dead embryo were removed. Eggs containing a viable embryo were redistributed over the same 4 incubators (2 replicates / treatment group). Two incubators were then maintained at an EST of 37.8°C, whereas the other two incubators were maintained at an EST of 36.7°C. EST control in all incubators was performed as described above.

At E18, all eggs were candled again and eggs containing a viable embryo were transferred to hatching baskets and placed back in the same incubators. After 454 h of incubation (E18h22), the incubator air temperatures were fixed at their current setting and EST was allowed to change.

Throughout incubation, relative humidity was maintained between 50% and 65% and CO2 levels were <3,500 ppm for all treatments. Eggs were turned every hour by an angle of 90° from the start of incubation until E18.

Hatch

From 468 h of incubation onwards (E19h12), every 6 h the four incubators were opened to check whether chicks had hatched. Hatched chicks were marked with a permanent marker on the head. Six hours later, these marked chicks were collected, feather sexed, and chick quality was scored as described in the section 'data collection'. Per treatment, every 8th chick that hatched was decapitated and organs were sampled as described below. Remaining chicks received a leg ring and were transferred to another incubator where they were housed in hatcher baskets in which they had ad libitum access to feed and water. As a result, each chick had access to feed and water within 12 h after emergence from the eggshell. In this incubator, continuous light was provided and temperature and relative humidity were maintained at 33.2°C and 50%, respectively. All chicks remained in the incubator until the last chick had hatched, which was at 519 h after the start of incubation (E21h15).

Grow-out

After all chicks had hatched, 40 male and 40 female first grade chicks per treatment were transported to the grow-out facility, which was located in a neighbouring building at 20 m distance. Chicks were divided over 32 floor pens in 2 adjacent similar broiler houses (8 replicate pens per treatment). Within each house, pens were divided over 4 blocks and within each block, treatments were randomly assigned. Each pen contained 5 male and 5 female broilers and from each sex, one broiler hatched early in the hatch window, 3 broilers were mid hatchers, and 1 broiler hatched late (defined as 12% / 76% / 12% of the total hatch window for early, mid, and late hatchers, respectively). Pen size was 1 x 2 m and each pen contained one Valènta feeding pan (VDL Agrotech, Eindhoven, the Netherlands), five drinking nipples

with drip cups, and one perch. The floor was covered with 1 cm thick layer of wood shavings. No extra wood shavings were added until slaughter age. Temperature and humidity were set according to the Ross 308 management guide. The moment that chicks were placed in the grow-out facility was regarded as d 1. The first three days after placement, 24 h of light was provided and thereafter a lighting schedule of 16 h of light : 8 h of darkness. Starter pellet feed (ME broiler = 2,850 kcal/kg, CP = 220 g/kg, digestible Lysine = 12.52 g/kg) with a diameter of 2.6 mm was provided from d 0 to d 10, grower pellet feed (ME broiler = 2,952 kcal/kg, CP = 209.7 g/kg, digestible Lysine = 11.81 g/kg) with a diameter of 3.2 mm was provided from d 10 to d 28, and finisher pellet feed (ME broiler = 2,999 kcal/kg, CP = 199.8 g/kg, digestible Lysine = 11.14 g/kg) with a diameter of 3.2 mm was provided from d 28 until slaughter age. Diets were made by Research Diet Services (RDS, Wijk bij Duurstede, the Netherlands) according to the guidelines of the Federation Dutch Animal Feed chain (CVB, 2016). Both on d 41 and on d 42, 64 broilers were slaughtered (16 broilers / treatment / day). Per pen, two male and two female broilers were randomly selected from the mid hatchers, electrically stunned, and decapitated. Broilers were not feed or water restricted prior to slaughter.

Data Collection

Each egg was weighed before the start of incubation and at transfer to the hatching baskets (E18) to calculate egg weight loss. Clear eggs and eggs containing a dead embryo at candling at E7, E15, and E18 were opened and scored for fertility or moment of mortality (Lourens et al., 2006). Hatchability was calculated as the number of hatched chicks divided by the number of eggs that contained a viable embryo at E7, right before treatments started. Hatch moment was calculated as the number of hours from start of incubation to emergence from the eggshell. Hatch window was calculated per treatment as the difference in hatch moment between the first and last chick per treatment. Neonatal chick quality was evaluated by determining chick weight, chick length from beak-tip to toe-tip, and navel quality score (Reijrink et al., 2009) as 1 (completely closed and clean), 2 (discolored and opened to a maximum of 2 mm), and 3 (discolored and opened to more than 2 mm). Neonatal chicks were classified as 2nd grade chicks if any abnormality (e.g. crossed beak, blindness, exposed brains, four legs, not incorporated yolk) was observed. All other neonatal chicks were classified as 1st grade chicks.

For the neonatal chicks that were decapitated, blood glucose was determined immediately after decapitation, using a blood glucose analyzer (Contour TS, Bayer AG, Leverkusen, Germany). Thereafter, chicks were opened and RY, heart, liver, intestines, and stomach were removed and weighed on a two-decimal scale. Stomach included the proventriculus, the intermediate zone, the ventriculus (gizzard) and the pylorus. Bursa of Fabricius and spleen were weighed on an analytical four-decimal scale (A200S, Sartorius GmbH, Göttingen, Germany). The gizzard was opened and erosions were scored as 0 (no erosions), 1 (single erosion), 2 (2 or 3 erosions), 3 (>3 erosions). Erosions were defined as a dark discolouration of the koilin layer (Gjevre et al., 2013). YBFM was calculated as neonatal chick weight minus RY weight.

During grow out, all broilers (n=320) were weighed individually at placement, d 7, 14, 21, 28, 35, and 40. Feed intake was also determined per pen on those weighing days. Feed conversion ratio (**FCR**) was calculated by dividing the amount of feed consumed by body weight gain in that particular period. FCR over the total grow out period was corrected for body weight at d 40 (**FCRc**) by adding body weight at d 40 as a covariate to the model (see section statistics).

Temperature preference was determined at d1 and d7 for half of the pens. Two male and two female broilers from the same pen were placed together in the middle of a wooden box (160 x 60 x 50 cm) with a Plexiglas lid and wood shavings at the bottom and two infrared lights (250 Watt) on one side of the box (creating a linear temperature gradient from 20 to 50°C). Twenty-four temperature sensors (Hobo UX100-011, Onset, Boune, United States) were equally distributed in the box at broiler height, which continuously monitored the actual temperature. Ten minutes after placement of the broilers in the box, the location of each broiler was noted and the ambient temperature of each location was determined, based on temperature of the sensor log data (protocol adapted from Walstra et al. (2010).

All broilers selected to be slaughtered (n=128) were weighed individually prior to slaughter (d 41 or d 42). After stunning, cutting, and bleeding, the head was removed (cut closest to upper cervical vertebra) and toes were removed (cut at tarsal joint) and thereafter the carcass was manually skinned and eviscerated. Heart, liver, intestines (incl. intestinal content), lungs, and stomach (incl. proventriculus, intermediate part, ventriculus, pylorus and feed content) were weighed. Legs (cut at hip joint), wings (cut at shoulder joint), and breast meat were removed and weighed (all without skin). The remaining carcass was weighed. The carcass yield was calculated for the eviscerated carcass (legs + wings + breast meat + remaining carcass) as a percentage of live body weight. The cut up yields and relative organ weights were calculated as a percentage of the eviscerated carcass.

Statistical analyses

All data was analyzed using the statistical software package SAS (Version 9.4, SAS institute 2010). The basic model used for all data was;

$$Y_{ij} = \mu + ESTwk2_i + ESTwk3_j + ESTwk2 \times ESTwk3_{ij} + e_{ij}, \quad [1]$$

where Y_{ij} = the dependent variable, μ = the overall mean, $ESTwk2_i$ = EST in wk 2 ($_i$=38.9 or 37.8°C), $ESTwk3_j$ = EST in wk 3 ($_j$=37.8 or 36.7°C), ESTwk2 x $ESTwk3_{ij}$ = the interaction between EST wk 2 and EST wk 3, and e_{ij} = the error term.

For all incubation variables, egg was considered the experimental unit and the egg tray number in which the egg was positioned was added as a random factor. For neonatal chick quality variables, hatch moment, and the temperature preference test, broiler was considered the experimental unit and sex was added to the model as a fixed factor. For chick length and

navel quality score, the person that scored the neonatal chick (3 individuals) was also added to the model as a fixed factor.

For post hatch performance variables and slaughter variables, pen was considered the experimental unit, the model described above was used, and block (1 to 8) was added as a random factor. Preliminary analysis showed that adding hatch moment (averaged per pen) to the model as a covariate had no significant effect for any performance variable, so it was excluded from the model. BW, ADG, ADFI, and FCR (all averaged / pen) were analyzed both per week and per total grow out period (d1 to 40) and FCRc was analyzed for the total grow out period by adding body weight at d 40 as a covariate to the model.

All variables were analysed with the PROC MIXED procedure in SAS, using the model described above, except for embryo mortality data, gizzard erosion score, and navel quality score. Embryo mortality data was analysed with the PROC GLIMMIX procedure, using the model described above, including a binomial log logit link function. Gizzard erosion score and navel quality score were also analysed with the PROC GLIMMIX procedure, using the model described above, including a multinomial log logit link function. In PROC MIXED procedure the model assumptions were verified by inspection of residual plots. All data was normal distributed, except for RY. RY was log-transformed and the LSMeans and the SEM of the untransformed data was used and given in the results section, whilst the *P*-value of the transformed data was given. Tukey adjustments for multiple comparisons were used to compare least square means (LSMeans). P-values ≤ 0.05 were considered to be significant.

RESULTS

Incubation

No interaction between EST wk 2 and EST wk 3 was found ($P \geq 0.52$) for any of the incubation variables, which includes egg weight loss, embryonic mortality (data not shown), hatchability (described below), and hatch moment (Table 1). Duration of the hatch window was 42 h for 37.8°C EST continuously, 36 h for 37.8 x 36.7°C EST for wk2 x wk3, respectively, and 30 h for both 38.9 x 37.8°C EST and 38.9 x 36.7°C EST for wk2 x wk3, respectively.

A higher EST of 38.9°C in wk 2 resulted in a higher egg weight loss compared to a constant EST of 37.8°C (10.3 vs 9.7 % ± 0.1 for 38.9°C and 37.8°C, respectively; $P<0.001$). Hatch moment was on average 5 h earlier when EST in wk 2 was raised to 38.9°C compared to a constant EST of 37.8°C ($P<0.001$; Table 1). EST in wk 2 had no effect on hatchability (95.7 vs 97.7 % ± 0.4 for 38.9°C and 37.8°C, respectively; $P=0.32$).

A lower EST of 36.7°C in wk 3 resulted in a 8 h later hatch moment ($P<0.001$; Table 1) compared to an constant EST of 37.8°C, but it had no effect on egg weight loss (9.9 vs 10.1 %

Table 1. Effect of two eggshell temperatures (**EST**; 37.8, 38.9°C) applied during wk 2 and two EST (36.7, 37.8°C) during wk 3 of incubation on hatch moment, neonatal chick quality, and relative organ weights at the moment of hatch.

Item	Hatch[1] moment (h)	BW[1] (g)	RY[2] (g)	YFBM[2] (g)	Chick[1] length (cm)	Navel[1] score (1-3)	Blood glucose[2] (mmol/L)	Heart[2] (%)[3]	Liver[2] (%)[3]	Bursa[2] (%)[3]	Spleen[2] (%)[3]	Intestines[2] (%)[3]	Stomach[2] (%)[3]	Giz.er.sc.[2] (0-3)
EST wk 2														
37.8°C	499[a]	46.04	5.76	40.17	19.4[b]	1.7	11.4[a]	0.84	2.63	0.0551	0.0280	4.62	5.57	1.9
38.9°C	494[b]	45.85	5.43	40.22	19.5[a]	1.7	11.0[b]	0.78	2.69	0.0505	0.0323	4.79	5.60	2.0
SEM	0.4	0.09	0.25	0.33	0.03	0.05	0.2	0.02	0.05	0.0066	0.0032	0.17	0.12	0.2
EST wk 3														
36.7°C	501[a]	45.93	5.49	40.20	19.4[b]	1.7	11.7[a]	0.85[a]	2.70	0.0618	0.0342	4.80	5.78[a]	1.9
37.8°C	493[b]	45.95	5.71	40.19	19.5[a]	1.7	10.7[b]	0.77[b]	2.62	0.0437	0.0260	4.61	5.39[b]	2.0
SEM	0.4	0.09	0.25	0.33	0.03	0.05	0.2	0.02	0.05	0.0066	0.0032	0.17	0.12	0.2
EST wk 2 x wk 3														
37.8 x 36.7°C	503	46.05	5.81	40.01	19.3	1.7	11.9	0.89	2.69	0.0646	0.0303	4.62	5.73	2.1
37.8 x 37.8°C	496	46.02	5.72	40.32	19.4	1.6	11.0	0.79	2.57	0.0454	0.0256	4.62	5.41	1.8
38.9 x 36.7°C	498	45.82	5.17	40.39	19.4	1.7	11.6	0.81	2.71	0.0589	0.0382	4.98	5.83	1.9
38.9 x 37.8°C	491	45.88	5.70	40.05	19.5	1.7	10.4	0.75	2.68	0.0420	0.0264	4.60	5.37	2.1
SEM	0.6	0.13	0.35	0.47	0.04	0.07	0.2	0.03	0.08	0.0093	0.0045	0.24	0.17	0.3
P-value														
wk 2	<0.001	0.14	0.39	0.90	<0.001	0.57	0.04	0.07	0.41	0.63	0.34	0.47	0.86	0.96
wk 3	<0.001	0.87	0.44	0.98	0.02	0.36	<0.001	0.008	0.33	0.06	0.08	0.41	0.03	0.86
wk 2 x wk 3	0.90	0.71	0.33	0.50	0.93	0.68	0.71	0.55	0.59	0.91	0.44	0.45	0.70	0.12

[1] n=103, n=104, n=111, n=108 for treatment groups 37.8 x 37.8°C, 38.9 x 37.8°C, 37.8 x 36.7°C, 38.9 x 36.7°C, respectively; determined within 12 h after emergence from the eggshell.

[2] RY= residual yolk, YFBM= yolk free body mass, mmol/L=millimole/L, Giz.er.sc.=gizzard erosion score: n=13, n=12, n=13, n=12 for treatment groups 37.8 x 37.8°C, 38.9 x 37.8°C, 37.8 x 36.7°C, 38.9 x 36.7°C, respectively; determined between 6 and 12 h after hatching. Stomach includes the proventriculus, intermediate part, pylorus and ventriculus.

[3] Weight relative to YFBM.

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

± 0.1 for 36.7 and 37.8°C, respectively; *P*=0.17) and on hatchability (97.2 vs 96.5 % ± 0.4 for 37.8°C and 36.7°C, respectively; *P*=0.67).

Hatch moment was on average 3 h earlier for female than for male chicks (495 vs 498 h ± 0.4, respectively; *P*<0.001).

Chick quality at hatch

No interaction between EST wk 2 and EST wk 3 was found for any of the chick quality variables at hatch (Table 1; *P*≥0.12). A higher EST of 38.9°C in wk 2 resulted in a 1 mm longer chick length (*P*<0.001) and 0.4 millimole/L lower blood glucose level (*P*=0.04) compared to a constant EST of 37.8°C. EST in wk 2 had no effect on BW, RY, YFBM, navel quality score, any organ weights relative to YFBM, or gizzard erosions (*P*≥0.07).

A lower EST of 36.7°C in wk 3 resulted in a 1 mm shorter chick length (*P*=0.02), 1.0 millimole/L higher blood glucose level, 0.08% higher relative heart weight (*P*=0.008), and a 0.39% higher relative stomach weight (*P*=0.03) compared to a constant EST of 37.8°C. EST in wk 3 had no effect on BW, RY, YFBM, navel quality score, relative liver, bursa, spleen, intestine weight, and gizzard erosions (*P*≥0.06).

Chick length was longer for male (19.4 cm) than for female chicks (19.2 cm; ±0.03; *P*=0.002). Navel quality score was higher (worse) for female (score 1.8) than for male chicks (score 1.6; *P*=0.03). Gizzard erosion score was higher (worse) for male (score 2.6) than for female chicks (score 1.6; *P*<0.001).

Temperature preference

Temperature preference of broilers at d1 and d7 did not show an interaction between EST in wk 2 and EST in wk 3 and was also not affected by a main effect of EST in wk 2 or EST in wk 3 (Table 2; *P*≥0.09). Temperature preference did not differ between male and female broilers at d 1 (*P*=0.81; ± 0.6) or d 7 (*P*=0.81; ± 0.2).

Table 2. Effect of two eggshell temperatures (**EST**; 37.8, 38.9°C) applied during wk 2 and two EST (36.7, 37.8°C) during wk 3 of incubation on broiler temperature preference (°C) at d 1 and d 7 post hatch.

Item	n[1]	d 1	d 7
EST wk 2			
37.8°C	32	33.9	27.4
38.9°C	32	33.4	27.2
SEM		0.6	0.2
EST wk 3			
36.7°C	32	34.4	27.4
37.8°C	32	33.0	27.2
SEM		0.6	0.2
EST wk 2 x EST wk 3			
37.8 x 36.7°C	16	35.1	27.3
37.8 x 37.8°C	16	32.7	27.6
38.9 x 36.7°C	16	33.6	27.5
38.9 x 37.8°C	16	33.2	26.8
SEM		0.8	0.3
P-value			
wk 2		0.53	0.32
wk 3		0.09	0.37
wk 2 x wk 3		0.26	0.09

[1] Number of broilers, 50:50 sex ratio.

Broiler performance

No interaction between EST wk 2 and EST wk 3 was found for any of the performance variables ($P \geq 0.12$), which includes BW, ADG, ADFI, FCR, and FCRc. A higher EST of 38.9°C in wk 2 compared to a constant EST of 37.8°C resulted in a 3 g higher BW at d1 (Table 3; $P < 0.001$), but BW at later ages was not affected by EST in wk 2 (Table 3; $P \geq 0.16$). A higher EST of 38.9°C in wk 2 compared to a constant EST of 37.8°C resulted in 3 g lower ADG in wk 5 (Table 4; $P = 0.05$), but it had no effect on ADG at other ages (Table 4; $P \geq 0.21$). ADFI was not affected at any age by EST in wk 2 ($P \geq 0.14$). A higher EST of 38.9°C in wk 2 compared to a constant EST of 37.8°C resulted in a 0.06 lower (better) FCR in week 6 (Table 4; $P = 0.04$), but FCR was not affected at other ages (Table 4; $P \geq 0.12$). Over the total grow out period (d1 to 40), EST in wk 2 had no effect on ADG, ADFI, FCR, and FCRc (Table 4; $P \geq 0.22$).

Table 3. Effect of two eggshell temperatures (**EST**; 37.8, 38.9°C) applied during wk 2 and two EST (36.7, 37.8°C) during wk 3 of incubation on later life broiler body weight (g).

Item	n[1]	d 1	d 7	d 14	d 21	d 28	d 35	d 40
EST wk 2								
37.8°C	16	53[b]	188	513	1,034	1,706	2,507	3,086
38.9°C	16	56[a]	190	511	1,042	1,707	2,486	3,053
SEM		0.3	2	4	8	13	18	24
EST wk 3								
36.7°C	16	53[b]	184[b]	501[b]	1,020[b]	1,677[b]	2,454[b]	3,023[b]
37.8°C	16	56[a]	193[a]	523[a]	1,056[a]	1,735[a]	2,539[a]	3,116[a]
SEM		0.3	2	4	8	13	18	24
EST wk 2 x EST wk 3								
37.8 x 36.7°C	8	51	183	499	1,010	1,663	2,454	3,040
37.8 x 37.8°C	8	56	192	527	1,057	1,748	2,560	3,132
38.9 x 36.7°C	8	54	186	504	1,029	1,691	2,453	3,006
38.9 x 37.8°C	8	57	194	518	1,054	1,722	2,518	3,100
SEM		0.4	2	5	12	18	25	35
P-value								
wk 2		<0.001	0.16	0.74	0.51	0.96	0.40	0.35
wk 3		<0.001	<0.001	<0.001	0.006	0.005	0.003	0.02
wk 2 x wk 3		0.15	0.79	0.18	0.35	0.16	0.42	0.99

[a-b] Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$).
[1] Number of pens, each containing 10 broilers, 50:50 sex ratio.

A lower EST of 36.7°C in wk 3 compared to a constant EST of 37.8°C resulted in a lower BW at all ages (Table 3; $P \leq 0.02$). A lower EST of 36.7°C in wk 3 compared to a constant EST of 37.8°C resulted in a lower ADG during wk 1, wk 2, and wk 4 (Table 4; $P \leq 0.03$), but comparable ADG in wk 3, wk 5, and wk 6 (Table 4; $P \geq 0.09$). ADFI was lower in wk 1 and wk 3 for a lower EST of 36.7°C in wk 3 compared to a constant EST of 37.8°C (Table 4; $P < 0.02$), but ADFI at other ages was not affected by EST in wk 3 (Table 4; $P \geq 0.06$). A lower EST of 36.7°C in wk 3 compared to a constant EST of 37.8°C resulted in a 0.02 lower (better) FCR in wk 1 (Table 4; $P = 0.04$), but in a higher (worse) FCR in wk 2 and wk 4 (Table 4; $P \leq 0.03$). In wk 3, wk 5, and wk 6 FCR was not affected by EST wk 3 (Table 4; $P \geq 0.20$). Over the total grow out period (d1 to 40), a lower EST of 36.7°C in wk 3 compared to a constant EST of 37.8°C resulted in a 3 g lower ADG (Table 4; $P = 0.02$) and a 3 g lower ADFI (Table 4; $P = 0.04$), whereas no effects were found for FCR and FCRc (Table 4; both $P = 0.94$).

Table 4. Effect of two eggshell temperatures (**EST**; 37.8, 38.9°C) applied during wk 2 and two EST (36.7, 37.8°C) during wk 3 of incubation on ADG, ADFI, and feed conversion ratio (**FCR**) of broilers per week and over the total grow out period (d 1 to 40).

Item	n	ADG							ADFI							FCR							
		week							week							week							
		1	2	3	4	5	6	total	1	2	3	4	5	6	total	1	2	3	4	5	6	total	corr[2]
EST wk 2																							
37.8°C	16	22	46	74	96	114[a]	114	78	24	59	98	146	178	201	117	1.08	1.28	1.33	1.53	1.56	1.77[a]	1.51	1.51
38.9°C	16	22	46	76	95	111[b]	114	77	25	58	99	146	174	194	115	1.10	1.26	1.31	1.53	1.58	1.71[b]	1.50	1.50
SEM		0.2	0.5	0.9	0.9	1.5	2.1	0.6	0.2	0.6	1.2	1.4	1.8	2.9	1.0	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01
EST wk 3																							
36.7°C	16	22[b]	45[b]	74	94[b]	111	112	76[b]	24[b]	58	96[b]	145	174	194	115[b]	1.08[b]	1.28[a]	1.30	1.55[a]	1.57	1.73	1.51	1.51
37.8°C	16	23[a]	47[a]	76	97[a]	114	116	79[a]	25[a]	59	101[a]	147	178	202	118[a]	1.10[a]	1.25[b]	1.33	1.51[b]	1.56	1.75	1.51	1.51
SEM		0.2	0.5	0.9	0.9	1.5	2.1	0.7	0.2	0.6	1.2	1.4	1.8	2.9	1.0	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01
EST wk 2 x EST wk 3																							
37.8 x 36.7°C	8	22	45	73	93	113	113	77	24	58	96	144	175	198	115	1.07	1.29	1.32	1.55	1.55	1.77	1.50	1.50
37.8 x 37.8°C	8	23	48	76	99	116	115	79	25	60	101	149	181	203	119	1.10	1.26	1.33	1.51	1.57	1.78	1.51	1.52
38.9 x 36.7°C	8	22	46	75	95	109	112	76	24	58	97	146	173	189	114	1.09	1.27	1.29	1.55	1.59	1.69	1.51	1.51
38.9 x 37.8°C	8	23	47	77	95	112	117	78	25	59	101	145	175	200	117	1.11	1.24	1.32	1.52	1.56	1.73	1.50	1.50
SEM		0.3	0.7	1.2	1.2	1.9	2.8	0.9	0.3	0.8	1.7	1.9	2.6	4.1	1.4	0.01	0.01	0.02	0.01	0.02	0.03	0.01	0.01
P-value																							
wk 2		0.48	0.79	0.21	0.45	0.05	0.79	0.30	0.21	0.34	0.73	0.66	0.15	0.14	0.22	0.12	0.15	0.29	0.65	0.23	0.04	0.52	0.50
wk 3		0.005	0.007	0.10	0.03	0.09	0.21	0.02	<0.001	0.15	0.02	0.34	0.14	0.06	0.04	0.04	0.009	0.20	0.03	0.70	0.44	0.94	0.94
wk 2 x wk 3		0.48	0.19	0.55	0.08	0.92	0.62	0.95	0.78	0.42	0.68	0.14	0.38	0.44	0.44	0.80	0.79	0.79	0.65	0.12	0.68	0.12	0.13

[a-b] Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$).
[1] Number of pens, each containing 10 broilers, 50:50 sex ratio.
[2] FCRcorrected for BW at d 40.

Slaughter

No interaction between EST wk 2 and EST wk 3 was found for any of the slaughter variables (Table 5; $P \geq 0.05$). A higher EST of 38.9°C in wk 2 resulted in higher relative stomach weight compared to a constant EST of 37.8°C (Table 5; Δ=0.2%; $P=0.009$). EST in wk 2 had no effect on carcass yield, cut up yields, or relative heart, liver, lungs, or intestines weight (Table 5; $P \geq 0.09$).

A lower EST of 36.7°C in wk 3 resulted in a lower relative breast meat yield compared to a constant EST of 37.8°C (Table 5; Δ=0.7%; $P=0.05$). Relative intestine weight was higher after a lower EST of 36.7°C in wk 3 compared to a constant EST of 37.8°C (Table 5; Δ=0.4%; $P=0.02$).

Table 5. Effect of two eggshell temperatures (**EST**; 37.8, 38.9°C) applied during wk 2 and two EST (36.7, 37.8°C) during wk 3 of incubation on broiler slaughter (d 41/d 42) variables.

Item	n[1]	Carcass yield[2]	Breast[3]	Legs[3]	Wings[3]	Heart[3]	Liver[3]	Lungs[3]	Intestines[3]	Stomach[3]
EST wk 2										
37.8°C	16	65.8	38.8	29.6	8.6	0.7	3.2	0.5	7.9	1.7[b]
38.9°C	16	65.9	39.2	29.5	8.6	0.7	3.1	0.6	7.9	1.9[a]
SEM		0.3	0.3	0.2	0.2	0.01	0.04	0.03	0.1	0.04
EST wk 3										
36.7°C	16	65.5	38.6[b]	29.6	8.6	0.7	3.2	0.6	8.1[a]	1.8
37.8°C	16	66.2	39.3[a]	29.5	8.6	0.7	3.2	0.6	7.7[b]	1.8
SEM		0.3	0.3	0.2	0.2	0.01	0.04	0.03	0.1	0.04
EST wk 2 x wk 3										
37.8 x 36.7°C	8	65.8	38.7	29.4	8.4	0.7	3.2	0.5	8.1	1.8
37.8 x 37.8°C	8	65.8	38.8	29.7	8.8	0.7	3.2	0.5	7.7	1.7
38.9 x 36.7°C	8	65.2	38.5	29.7	8.8	0.7	3.2	0.6	8.1	1.8
38.9 x 37.8°C	8	66.6	39.8	29.3	8.4	0.7	3.1	0.6	7.6	2.0
SEM		0.4	0.4	0.3	0.2	0.02	0.06	0.03	0.2	0.1
P-values										
wk 2		0.80	0.21	0.71	0.95	0.76	0.34	0.09	0.79	0.009
wk 3		0.08	0.05	0.93	0.95	0.14	0.70	0.85	0.02	0.66
wk 2 x wk 3		0.08	0.08	0.23	0.04[4]	0.37	0.99	0.55	0.85	0.09

[a-b] Least squares means within a column and factor lacking a common superscript differ ($P \leq 0.05$).
[1] Number of pens, each containing 10 broilers, 50:50 sex ratio.
[2] % relative to live body weight.
[3] % relative to carcass weight. Stomach includes proventriculus, intermediate part, pylorus, and ventriculus.
[4] After Tukey adjustment no significant differences appeared.

DISCUSSION

It was hypothesized that an incubation pattern consisting of a higher EST of 38.9°C in the second week of incubation in combination with a lower EST of 36.7°C in the last week of incubation would result in most optimal embryo development, neonatal chick quality, and subsequent broiler performance during grow out compared to a constant EST of 37.8°C throughout incubation. This study showed that this incubation temperature pattern had no synergistic stimulating effect on neonatal chick quality or on broiler grow out performance compared to a constant EST of 37.8°C in the second and third week of incubation, as there was no interaction between EST in wk 2 and EST in wk 3 on any of the neonatal chick quality and grow out variables.

Increasing the EST in wk 2 of incubation to 38.9°C compared to maintaining a constant EST of 37.8°C affected embryo development. A smaller hatch window (12h) and earlier hatch moment (5h) after a higher incubation temperature of 38.9°C EST in wk 2 compared to a constant EST of 37.8°C suggests that embryo development was accelerated. On the one hand, this accelerated embryo development seemed beneficial, because chick length at hatch was 1 mm longer when EST was increased in wk 2 of incubation. It is known that in wk 2 of incubation ossification of the long leg bones occurs and that temperature has an effect on ossification speed (Rommel et al., 2001; Mackie et al., 2008;). A faster ossification may have resulted in longer bones and consequently a slightly longer chick. Studies have shown that chick length at hatch is positively correlated to broiler BW at slaughter age (Wolanski et al., 2004, Molenaar et al., 2008; Petek et al., 2010), although the relationship was not very strong (max r = 0.60) and other studies did not confirm this (Willemsen et al., 2008). On the other hand, this accelerated embryo development seemed to be accompanied with a lower blood glucose level at hatch. Lower blood glucose levels at hatch might be explained by: 1) less hepatic glycogen was available to the embryo to turn into blood glucose. Perhaps hepatic glycogen storage by the embryo was impaired in wk 2 due to the higher EST. Studies showed that a higher EST than 37.8°C resulted in lower hepatic glycogen stores (Willemsen et al., 2010, Molenaar et al., 2011b, Maatjens et al., 2014a), but all these studies were performed with a high temperature until the end of incubation or 2) more glucose was utilized by the embryo, for instance during the hatching process. Blood glucose serves as the main source for adenosine triphosphate (ATP) to provide energy (Freeman, 1965), especially between external pipping and hatch, as this is an energy demanding process (De Oliveira et al., 2008). The duration between external pipping and hatch may have taken longer for chicks incubated at a higher EST of 38.9°C in wk 2, for instance because these chicks were slightly longer or because egg weight loss during incubation was higher in this treatment group.

EST in wk 2 had no effect on broiler grow out performance or slaughter yield. A higher EST of 38.9°C in wk 2 only increased broiler BW at d1, but this was most likely due to a difference in hatching moment. Body weight and YFBM at hatch were similar between

both groups, but after hatch all chicks had immediate access to feed and water and broilers incubated at 38.9°C EST in wk 2 hatched on average 5h earlier and therefore had on average 5h longer between hatch and moment of weighing at d1 in which they could eat and grow.

Lowering the EST in wk 3 of incubation to 36.7°C compared to maintaining a constant EST of 37.8°C was hypothesized to optimize the balance between metabolic rate and available oxygen at embryo level (Nangsuay et al., 2016) and thereby improving nutrient oxidation for embryonic development (Maatjens et al., 2016a, 2017). A lower EST of 36.7°C in the last week of incubation resulted on the one hand in a higher relative heart and stomach weight and higher blood glucose level at hatch, which suggest that oxygen availability and embryonic development was indeed stimulated. On the other hand, a shorter chick length was found, which may suggest that embryonic development was retarded. The higher blood glucose level at hatch that was found might be explained by a lower metabolic rate that will probably occur when EST is lowered in wk 3. A lower metabolic rate results in a lower demand for oxygen and if the demand does not exceed the available amount of oxygen, which is limited during late incubation due to eggshell conductance, the embryo will probably use mostly fat as energy resource. In case oxygen availability is insufficient, for instance when metabolic rate is increased, the embryo might also use glucose via glycolysis as an energy resource, which is an anaerobic process. The higher relative heart and stomach weights that were found in the current study when EST was lower in wk 3 might also be caused by an improved balance between metabolic rate and available oxygen and thereby improved resources available for development of these organs. This is supported by a study from Wineland et al. (2001) who were the first to show that a lower incubation temperature during late incubation resulted in a higher heart weight. Molenaar et al. (2011b) showed that increasing oxygen levels to 25% from E7 to E19 of incubation resulted in a higher YFBM when EST was 38.9°C in that period, but relative organ weights were not affected. This indicates that oxygen was available to the embryo, but that organ development was not affected by a higher oxygen availability. Probably the higher relative heart and stomach weights that were found in the current study were not the result of a higher oxygen availability, but were the result of a difference in incubation duration. The total incubation duration was 8 h longer for embryos in the lower EST treatment group and therefore these embryos had 8 h more time within the egg to develop. As the maturation of organs mainly takes place during late incubation, this prolonged incubation duration might specifically have affected relative organ weights and for instance not the length of the chick.

The higher blood glucose level and the higher relative stomach weight and heart weight that were found at hatch after a lower EST in wk 3 suggest that some parts of embryonic development were stimulated after a lower EST in wk 3 compared to a constant EST of 37.8°C. Some chick quality characteristics seemed improved and as a result a higher broiler grow out performance would be expected. After all, a worse chick quality at hatch that was found after a higher EST of 38.9°C in wk 3 was found to decrease performance upon slaughter age (Hulet et al., 2007; Leksrisompong et al., 2007; Ipek et al., 2014; Maatjens et al., 2016b), so it can

be speculated that a better chick quality at hatch that was found in the current study after a lower EST of 36.7°C in wk 3 could improve performance upon slaughter age. For instance, the higher blood glucose level at hatch indicates that more energy was available during the start of life and the higher relative stomach weight could indicate an improved development of the digestive tract. Furthermore, concerning the higher relative heart weight, it can be suggested that during grow out with a high growth rate, a larger heart might improve provision of oxygen and nutrients to the tissues and consequently broiler grow out performance might be improved (Molenaar et al., 2011a; Sozcu and Ipek, 2015; Druyan et al., 2018;). However, grow out performance at later ages showed opposite results. It seems that improved chick quality characteristics at hatch after a lower EST in wk 3 are only short term. For instance, FCR was improved in wk 1, but not at later ages for broilers incubated at a lower EST of 36.7°C in wk 3 compared to a constant EST of 37.8°C. Maybe the higher relative stomach and heart weight at hatch after a lower EST in wk 3 of incubation were not the result of an 8 h longer incubation duration and maturation of organs as stated before, but of a breakdown of organ muscles in the constant EST treatment group. The breakdown of own body muscles is hypothesized to be a short-term solution to gain glycogen via gluconeogenesis in anearobic circumstances (Maatjens et al., 2017) and it is known that oxygen is limited in wk 3 of incubation. Muscle break down during incubation was probably recovered quickly after hatch and probably therefore the higher relative organ weights at hatch after a lower EST in wk 3 did not stimulate broiler performance during grow out. In the current study, relative organ weights at slaughter age did not differ anymore between incubation temperatures and Maatjens et al. (2016b) also found that differences in relative heart weights that were found at hatch after different incubation temperatures disappeared within 1 wk after hatching. Nevertheless, no indicators of fewer muscle degradation were found in the blood of chicks at hatch that were incubated at lower EST of 36.7°C during late incubation compared to a constant EST of 37.8°C, because levels of lactate and uric acid were similar (Maatjens et al., 2017). Therefore muscle degradation seems not very likely and it remains unknown why broiler performance during grow out was not higher, whilst some chick quality characteristics at hatch were improved after a lower EST of 36.7°C in wk 3 compared to a constant EST of 37.8°C. It can be speculated that broilers prefer different conditions in the growing house after different incubation temperatures, but this was not illustrated in the temperature preference test of the current study.

A lower EST in wk 3 of 36.7°C negatively affected broiler grow out performance upon slaughter age. Broiler BW was lower at all ages and ADG and ADFI were lower over the total grow out period when EST in wk 3 of incubation was lowered to 36.7°C compared to maintaining EST at 37.8°C. Most likely, grow out performance was lower, because the onset of growth was delayed due to a longer incubation duration and thus a later moment of hatch and a shorter timespan with access to exogenous feed and water in the 36.7°C EST treatment group. Chicks from the 36.7°C EST in wk 3 treatment group hatched on average 8 h later compared to chicks from the 37.8°C EST treatment. All broilers had access to ad libitum feed

and water within 12 h after hatch and were placed in the growing houses at the same time point, indicating that broilers incubated at an EST of 36.7°C had on average 8 h less to grow between hatch and placement compared to broilers incubated at an EST of 37.8°C. This indeed could have had an effect, as BW at hatch was similar for both EST wk 3 treatment groups, whilst it was significantly lower at d 1 for the 36.7°C EST treatment group than for the 37.8°C EST treatment group. Moreover, extrapolation of BW data also suggests that onset of growth was delayed after incubation at 36.7°C EST in wk 3, without affecting growth competence. Fitting a polynomial curve to the body weight data of the 36.7°C EST treatment (R^2>0.99) and extrapolating it to 40 d and 8 h (40.333 days) results in a predicted BW of 3,105 g. This only differs 11 g from the actual average BW of the constant 37.8°C EST treatment (3,116 g). Additionally, not only the average incubation time differed between the 36.7°C EST treatment in wk 3 and the constant 37.8°C EST treatment in wk 3, but also the duration of the hatch window. The hatch window of the constant 37.8°C EST treatment was 42 h, whilst the hatch window of the 36.7°C EST treatment was only 30 h. As a result, some broilers from the constant 37.8°C EST treatment, especially the early hatchers, were even more ahead in growth than the average 8 h compared to broilers from to the 36.7°C EST treatment. Besides, FCR over the total grow out period and slaughter yields were not different between broilers incubated at a lower EST of 36.7°C EST in wk 3 and broilers incubated at a constant EST of 37.8°C, which suggests that growth efficiency was not affected and that a delayed onset of growth was more likely to be the explanation in growth performance differences between EST in wk 3.

In conclusion, the hypothesis that a higher EST of 38.9°C in wk 2 of incubation in combination with a lower EST of 36.7°C EST in wk 3 of incubation would stimulate embryo development and grow out performance compared to a constant EST of 37.8°C could not be accepted. EST in wk 2 did not interact with EST in wk 3 for any of the neonatal chick quality or grow out variables. A higher EST of 38.9°C in wk 2 compared to a constant EST of 37.8°C had little effect on neonatal chick quality and grow out performance was not affected. A lower EST 36.7°C in wk 3 compared to incubation at a constant EST of 37.8°C seemed to stimulate neonatal chick quality in terms of some relative organ weights, but grow out performance was not higher. Thus, so far there is no good reason to deviate from the standard of incubating eggs at a constant EST of 37.8°C throughout incubation.

ACKNOWLEDGMENTS

The authors wish to thank HatchTech for funding this project, Lagerwey hatchery for providing the eggs and the animal caretakers, MSc students (Lara olde Bolhaar, Ilonka van der Wagt, Kelly Hoogkamer), WUR colleagues (Bjorge Laurenssen, Marcel Heetkamp, Ilona van den Anker-Hensen, Monique Ooms, Henny Reimert, Bahadir Can Güz, Marieke van Os-Priester), and HatchTech colleague Gerald Aalbers for their assistance.

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CHAPTER 3

EFFECTS OF EGGSHELL TEMPERATURE PATTERN DURING INCUBATION ON PRIMARY IMMUNE ORGAN DEVELOPMENT AND BROILER IMMUNE RESPONSE IN LATER LIFE

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Poultry Science (2020) 99, 6619 – 6629

ABSTRACT

Eggshell temperature (**EST**) during incubation greatly affects embryo development, chick quality at hatch, and subsequently various broiler physiological systems. Until now, a constant EST of 37.8°C seems optimal. Data on effects of EST patterns on immune organ development and subsequent broiler immune response are, however, scarce. A higher EST of 38.9°C in week 2 and/or a lower EST of 36.7°C in week 3 of incubation potentially positively affect embryo immune organ development and broiler immune response post hatch. Broiler eggs (n=468) were incubated at 4 different EST patterns (n=117 eggs / treatment) from week 2 of incubation onwards. EST week 1 (embryonic age (**E**)0 < E7) was 37.8°C for all eggs. EST week 2 (E7 < E14) was either 37.8°C (**Control**) or 38.9°C (**Higher**) and EST week 3 (E14 - hatch) was either Control or 36.7°C (**Lower**). At hatch, histology of bursal follicles and jejunum villi and crypts were determined as well as heterophil to lymphocyte ratio (**H:L**) (n=49). Post hatch, both sexes were grown in 8 pens/treatment for 6 weeks (n=320). Natural antibodies (**NAb**) were determined at day 14, 22, and slaughter (day 41 or 42) as an indicator of immunocompetence and response to a Newcastle disease (**NCD**) vaccination was determined by antibody levels at day 22 and slaughter (n=128). Results showed no interaction EST week 2 x EST week 3, except for jejunum histology. Higher EST in week 2 resulted in lower cell density within bursal follicles (P=0.02) and a tendency for lower H:L (P=0.07) at hatch and higher NCD titres at slaughter (P=0.02) than Control EST. Lower EST in week 3 resulted at hatch in higher cell density within bursal follicles, higher H:L (both P<0.05) and a tendency for a higher post hatch mortality rate than Control EST (P=0.10). In conclusion, Higher EST in week 2 during incubation may benefit embryonic immune organ development and post-hatch broiler immunocompetence, whilst Lower EST in week 3 showed opposite indications.

INTRODUCTION

Infectious diseases in broiler husbandry impair broiler health and welfare and cause major economic losses (Jones et al., 2019). Currently, medicines are frequently used to prevent or cure infectious diseases. However, the use of medicines brings costs and comes with the risk of antimicrobial resistance (**AMR**), which is an increasing worldwide threat to animal and human health (ECDC, 2018). Thus, other strategies rather than the usage of medicines are needed in reducing the incidence of infectious diseases in broiler husbandry.

A well-functioning immune system is essential for broilers to counteract infectious diseases. A promising strategy to enhance a broiler's immunocompetence is by stimulating their immune system already during embryonic development. Embryo development is mainly affected by embryo temperature. Embryo temperature is accurately (±0.2°C) reflected by eggshell temperature (**EST**) during incubation (French, 1997). It has been shown that EST has pronounced effects on embryo body weight, yolk uptake, and development of organs, such as the heart and liver (Lourens et al., 2007; Molenaar et al., 2010; Maatjens et al., 2014; Van der Pol et al., 2014; Nangsuay et al., 2017). Moreover, studies showed that EST can also affect development of primary immune organs. For instance, histology of the bursa, gastro intestinal tract, spleen, and thymus at hatch were affected by high (38.1-39.0°C) incubator temperatures (Oznurlu et al., 2010; Liu et al., 2013; Leandro et al., 2017). Therefore, adjustments in EST patterns can potentially improve embryo immune organ development. We hypothesize that improved embryo immune organ development, due to an optimized EST pattern, will affect later life immune functionality. Broilers might, for instance, have a higher production and proliferation of B-cells from their bursa to the peripheral blood. Additionally, invading pathogens can be detected more rapidly by lymphocytes and a rapid onset of phagocytosis and antibody production might occur. Consequently, deleterious effects of infectious diseases on broiler health and performance will be smaller without medical interference, due to quick and sufficient responses of the broiler's immune system.

Studies showed that in some avian species incubation temperature can indeed affect immune response in later life. For instance, in Peking ducks, wood ducks, tree swallows, or quail, incubation temperature manipulations affected humoral or cellular immune response up to 55 days post hatch (Ardia et al., 2010; DuRant et al., 2012; Liu et al., 2013; Burrows et al., 2019; Shanmugasundaram et al., 2019). For chickens, and especially for broilers, studies on the effect of incubation temperature on immune response in later life are limited and results are inconsistent. Santin et al. (2013) concluded that broiler immune response post hatch was not affected by incubation temperature. In this study, broilers were incubated at a constant incubator temperature of 37.8°C or at 1°C higher or lower from the 14th day of incubation onwards. No difference in antibody titers against Newcastle disease virus (**NCD**) or infectious bursal disease at day 14 or day 35 post hatch were found. Rajkumar et al. (2015) also concluded that broiler immune responses post hatch was not affected by incubation

temperature. In their study, broilers were incubated at a constant incubator temperature of 37.8°C or at a 2°C higher incubator temperature during 3 h at day 16, 17, and 18 of incubation. At day 42 post hatch no differences in NCD antibody titres or swelling reaction in the wattle to Phytohaemagglutinin-P injection were found. Increasing incubator temperature by 1°C from day 10 of incubation onwards compared to a constant incubator temperature of 37.8°C was found to increase mucin expression and reduce colonization of Salmonella Enteritidis in the cecal content of 10 day old broilers when inoculated with Salmonella Enteritidis at 2 days post hatch (De Barros Moreira Filho et al., 2005). Somewhat contradictory, the same thermal manipulation treatment by Oznurlu et al. (2010) resulted in lower ACP-ase positive peripheral blood lymphocytes percentage and a higher ratio of heterophils to lymphocytes in broiler chicks at 7 days post hatch.

It appears that incubation temperature can affect broiler immune response in later life, but the EST pattern that result in optimal immune response has probably not been found yet. All studies mentioned in the previous paragraph adjusted incubation temperature treatments based on incubator temperature and not on EST. Incubator temperature differs from EST and this difference varies between incubator systems due to a difference in capacity to transfer embryonic heat from the egg to the surrounding air (Meijerhof and Van Beek, 1993; Lourens et al., 2005, 2006). This may explain some of the differences found between previous studies. A constant EST of 37.8°C throughout incubation is considered optimal for chick quality at hatch in terms of body length and yolk free body mass (Lourens et al., 2005). However, recently it was shown that a higher EST of 38.9°C during week 2 of incubation compared to a constant EST of 37.8°C improved embryonic growth rate (Nangsuay et al., 2016) and resulted in a longer chick length at hatch (Wijnen et al., 2020) and improved tibia bone characteristics at slaughter age (Güz et al., 2020). Besides, a lower EST of 36.7°C during week 3 of incubation compared to a constant EST of 37.8°C was shown to result in a higher yolk-free body mass and higher relative weights of the heart, liver, stomach, intestines, spleen (tendency), and bursa (Maatjens et al., 2016a, b; Wijnen et al., 2020). These results suggest that a higher EST of 38.9°C during week 2 in combination with a lower EST of 36.7°C during week 3 of incubation may also affect embryo immune organ development and consequently post-hatch immune response, but this was not studied. Therefore, the aim of the current study was to investigate whether a higher EST in week 2 and/or a lower EST in week 3 of incubation affect primary immune organ development and post-hatch immune response in broilers.

MATERIAL AND METHODS

Experimental Design

Eggs were incubated in a 2 x 2 factorial arrangement at 37.8°C EST (**Control**) or at 38.9°C EST (**Higher**) during week 2 of incubation and at Control EST or 36.7°C EST (**Lower**) during

week 3 of incubation (n=117 eggs / treatment). The experimental protocol was approved by the Governmental Commission on Animal Experiments, the Hague, the Netherlands; approval number: 2016.W-0087.001. For further details about material and methods, please review Wijnen et al. (2020).

Incubation

Eggs (n=468) within a 3 gram weight range from the average egg weight (63.5 gram) of a 44 week old Ross 308 broiler breeder flock were used. Before the start of incubation, eggs were divided over 8 setter trays (58 or 59 eggs / tray) and warmed linearly in 14 h from storage temperature (20°C) to an EST of 37.8°C. The moment the eggs reached an EST of 37.8°C was considered to be the start of incubation (E0). During week 1 of incubation (E0 up to and including E6), all eggs were incubated in the same incubator at an EST of 37.8°C. During week 2 of incubation (E7 up to and including E13), egg trays were equally divided over 4 incubators (Figure 1). During week 3 of incubation (E8 up to and including hatch), each egg tray was divided over 2 new trays (29 or 30 eggs / tray) and trays were again mixed over 4 incubators according to their treatment (Figure 1). Air temperature within each incubator was continuously adjusted automatically based on the median EST of 4 EST sensors (dry bulb) per incubator (climate respiration chamber type – details provided by Heetkamp et al. (2015). Throughout the incubation period, relative humidity was maintained between 50% and 65% and CO_2 levels were <3,500 ppm. Eggs were turned 90° every hour from the start of incubation until embryonic day (**E**) 18.

	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
	EST (°C)			Traynumber		
Incubator A	37.8	37.8	36.7	1 to 8	1 & 3	3.1 & 4.1 & 7.1 & 8.1
Incubator B	empty	38.9	37.8	empty	5 & 7	1.1 & 2.1 & 5.1 & 6.1
Incubator C	empty	38.9	37.8	empty	6 & 8	1.2 & 2.2 & 5.2 & 6.2
Incubator D	empty	37.8	36.7	empty	2 & 4	3.2 & 4.2 & 7.2 & 8.2

Figure 1. Schematic overview of treatment (eggshell temperature; **EST**) and eggtrays allocation over 4 different incubators (A to D) during 3 weeks of incubation.

Hatch

From E19 h12 onward (468 h of incubation), each incubator was opened every 6 hours to mark newly hatched chicks. Six hours after a chick was marked it was collected from the incubator, feather sexed, and classified either as second-grade chick if any abnormality was observed (e.g., crossed beak, blindness, exposed brains, 4 legs, exposed yolk) or as first-grade chick (all remaining chicks). Every 8th first-grade chick that hatched per treatment was decapitated and dissected. This resulted in dissection of 12 chicks per treatment. All remaining

first-grade chicks (n=337) were provided immediately with feed and water (within 12 hours after hatch) and 40 chicks of each sex per treatment were selected for the grow-out period. For details about exact hatch moments, please review Wijnen et al. (2020).

Grow-out

Broilers were divided over 2 adjacent houses in 32 floor pens (8 replicate pens / treatment). Within each house, pens were allocated over 4 blocks. Within each block, all 4 treatments were randomly assigned. Each pen contained 5 male and 5 female broilers. Broilers were grown for 6 weeks. They were vaccinated against infectious bronchitis at day 1 (Nobilis MA5) and against NCD at day 15 (inactivated Nobilis Newcavac 1000d).

Data Collection

Organ development

From the dissection chicks at hatch (12 chicks / treatment), the entire bursa was collected as well as approximately 1.5 cm of the first part of the jejunum (right after the distal part of the duodenum). A Swiss roll was made from the jejunum as described by Molenbeek and Ruitenberg (1981). The bursa and intestine samples were fixated in 4% formaldehyde in PBS for 2 days and thereafter they were stored in 70% ethanol until processing (approx. 12 months after hatch). At processing, they were put in paraffin, sliced (jejunum 3 µm and bursa 7 µm) and mounted on a glass microscope slide. Slides were hematoxylin and eosin (HE) stained.

One slide per bursa was examined under the microscope (Olympus BX41) for fold and follicle appearance. The longest intact fold inside the bursa slice was photographed with a microscope camera (MC500-W 3rd gen.) on a 4x magnification (Figure 2A). The number of follicles inside this fold was counted with the use of Clip Studio Paint (Version 1.6.2, Figure 2A). Follicles that were damaged (e.g. cut in half, cell leakage, etc.) were not included. If the fold had a side branch in addition to the main branch (Figure 2B), both the main branch and the side branch were included. Occurrence of a side branch was equal for all treatments and seen in 16% of the slices. Thereafter, 10 individual follicles were selected randomly from each fold. These 10 follicles were photographed with a 10x magnification and analysed. Length and width were measured using the standard straight line tool (Clip Studio Paint, version 1.6.2; Figure 2C). Additionally, circumference was determined by creating a custom ruler to draw a continuous line around the follicles and measuring the length of the line (Figure 2C). Finally, follicle area and cell density within each follicle were determined by converting the follicle into a black and white image (Paint.Net; Figure 2D). To determine the area, all pixels within the circumference were counted. To determine the cell density (%), the number of black pixels were counted and divided by the total number of pixels. For all follicle characteristics, the average of the 10 follicles was calculated and these averages were used for the statistical analysis.

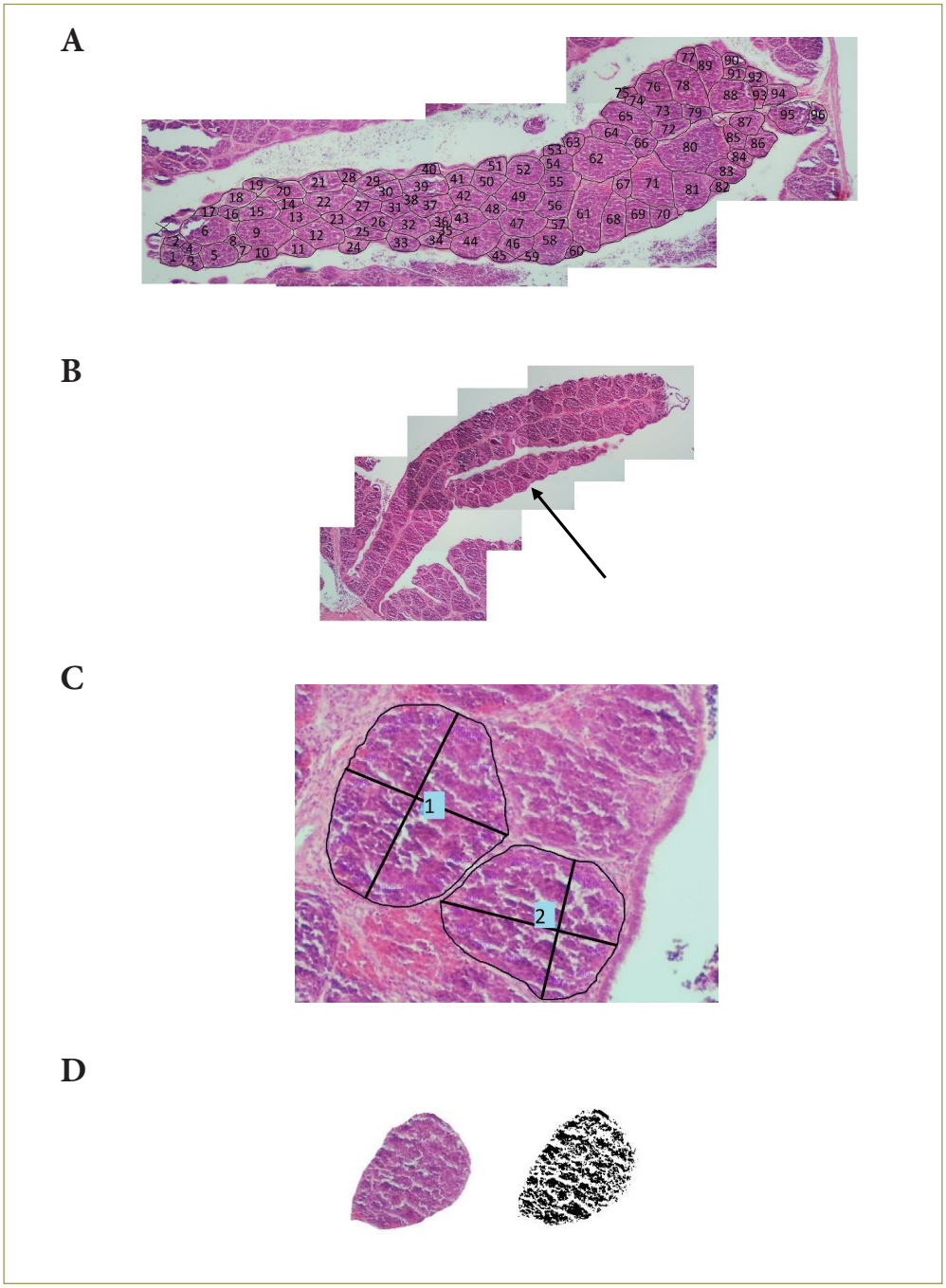

Figure 2. Bursa of Fabricius appearance of broiler chicks at hatch. Follicles within 1 fold were counted (**A**; n=96 in this example). Side branches were included (**B**). Follicle length, width, and circumference was measured (**C;** example 2 follicles). Cell density within follicles was determined by converting the image to black pixels and counting no. of black pixels (**D**).

Photomicrographs of 1 slide per jejunum sample were analysed on a microscope (Leica DM3000 LED) for villi and crypt appearance and histopathology, using Leica LAS V4.8 software. From each slide, 10 representative villi and associated crypts were randomly chosen. Villi lengths were measured as the distance (in μm) from the villus tip to the villus-crypt junction. Crypt depths were measured as the distance (in μm) from the base of the crypt to the villus-crypt junction (Uni et al., 1995). The averages of these 10 villi and crypts were calculated and these averages were used in statistical analysis. Histopathological examination was performed by a veterinarian and the absence or presence was scored of villi fusion, mucosal lymphocyte/plasma cell infiltration, mucosal heterophil infiltration, and enterocyte damage. For each variable, a score 0 was noted in case of absence and 1 in case of presence. All different pathologies were assumed equally bad and therefore they were summed to calculate a total histopathology score per slide, ending up in a score of minimum 0 and maximum 4.

H:L ratio

From the dissection chicks at hatch, 1 droplet of blood was collected after decapitation (mixture artery and vein) and a blood smear was made on a microscope slide. A May-Grünwald-Giemsma coloring was applied and smears were stored at room temperature until they were analyzed by light microscopy. A total of 100 true whole leukocytes (incl. heterophils, lymphocytes, monocytes, basophils, and eosinophils but excl. erythrocytes and thrombocytes) were counted on each slide and the heterophil to lymphocyte ratio (**H:L**) was calculated.

Newcastle Disease vaccination response

Antibody response to an inactivated NCD vaccine was determined as a measure of the acquired B-cell reactivity. From the dissection chicks at hatch, blood was collected at hatch via decapitation (mixture of arterial and venous blood). Additionally, from 128 randomly chosen broilers (2 males and 2 females / pen), blood was collected twice; once at 7 days post vaccination (day 22) via the wing vein and once at slaughter via decapitation (mixture arterial and venous blood). Slaughter was either on day 41 or on day 42. Each day, 1 male and 1 female broiler per pen were slaughtered (total of 32 broilers / treatment). Blood was collected in natrium heparinized tubes (Vacuette 4 ml FX, Greiner Bio-One), stored on ice, and plasma was collected after centrifugation at 2,000 x g for 10 minutes. Plasma was stored at -20°C until samples were analyzed for antibody titers for NCD with an ELISA kit (06-01096-15 IDEXX, Hoofddorp, the Netherlands). Briefly, in 96-well plates coated with NCD antigen the diluted plasma samples were dispensed as well as negative control serum (diluted chicken serum non-reactive to NDV preserved with sodium azide) and positive control serum (diluted chicken anti NCD serum). Well-plates were incubated for 30 minutes at 20°C, washed with deionized water, and goat anti chicken conjugate (HRPO preserved with gentamicin and proclin) was added. Plates were incubated again for 30 minutes at 20°C, washed, and TMB substrate was added. Plates were incubated for 15 minutes at 15°C at 20°C and stop solution was added.

Natural antibodies

At day 14, 22, and at slaughter (day 41 or 42) blood samples were taken and treated as described above. Plasma samples were analyzed for the level of natural antibodies (**NAb**) through the amount of immunoglobulin binding to keyhole limpet hemocyanin (**KLH**) as described for layer chickens by Berghof et al. (2015).

Mortality

Broilers that died were noted daily and mortality rates were calculated per pen relative to the number of broilers at placement. Four broilers were culled for human reasons (e.g. poor gait) and were excluded from the analysis.

Statistical analyses

All data was analyzed using the statistical software package SAS (Version 9.4, SAS institute, 2010). The variables determined in hatchlings were analysed using general linear model 1 (Proc Mixed – 3-way ANOVA):

$$Y_{ijk} = \mu + ESTwk2_i + ESTwk3_j + ESTwk2 \times ESTwk3_{ij} + sex_k + e_{ijk}, \quad [1]$$

where Y_{ijk} = the dependent variable, μ = the overall mean, $ESTwk2_i$ = EST in wk 2 ($_i$=38.9 or 37.8°C), $ESTwk3_j$ = EST in wk 3 ($_j$=37.8 or 36.7°C), ESTwk2 x $ESTwk3_{ij}$ = the interaction between EST wk 2 and EST wk 3, sex = sex ($_k$ = male or female), and e_{ijk} = the error term.

Preliminary statistical analysis did not show significant effects of sex x ESTwk2 or sex x ESTwk3 or sex x ESTwk2 x ESTwk3 for any of the variables ($P \geq 0.07$). Therefore, interactions between sex and EST were excluded from the final model.

The hatchling was used as the experimental unit for all hatchling variables. For post hatch variables, pen was used as the experimental unit. For mortality, model 1 was used, but without sex. For NCD vaccination response and natural antibodies, measurements were performed on individual broilers, but analyzed on pen basis, by extending model 1 with pen (1 to 32) nested within block (1 to 8) as a random factor. NAb titers were measured at 3 moments for the same broiler (day 14, 22, slaughter) and broiler was considered to be the repeated subject. Model 1 was extended with day and the interactions between day and ESTwk2, day and ESTwk3, day and sex. A compound symmetry covariance structure was assumed.

Model assumptions were verified by inspection of residual plots. All data was distributed normally. Tukey adjustments for multiple comparisons were used to compare least square means (LSMeans).

Histopathology score (0 – 4) of the intestines was analysed with a generalized linear model (Proc Glimmix), using model 1 and a multinomial cumulative logit link function.

RESULTS

Bursa of Fabricius

No interaction between EST week 2 and EST week 3 was found ($P \geq 0.10$) for any of the bursa characteristics (Table 1). Higher EST in week 2 resulted in a 7% lower cell density within the bursal follicles compared to Control ($P=0.024$). Lower EST in week 3 resulted in an 8% higher cell density within the bursal follicles compared to Control ($P=0.007$). Bursa characteristics did not differ between sexes ($P \geq 0.37$; data not shown).

Table 1. Effect of a higher eggshell temperature (**EST**) of 38.9°C during week 2 of incubation (**Higher**) and/or a lower EST of 36.7°C during week 3 of incubation (**Lower**) compared to a control EST of 37.8°C (**Control**) on histological characteristics of follicles within the Bursa of Fabricius and histology of the jejunum of broiler chicks at hatch.

	Bursa follicles						Jejunum	
Treatment	**Follicles/ fold[1] (no.)**	**Length[12] (px)**	**Width[12] (px)**	**Circumference[12] (px)**	**Area[12] (px)**	**Cell Density[12] (%)**	**Villi length[3] (µm)**	**Crypt depth[3] (µm)**
EST week 2								
Control	70	565	412	1,667	186,238	46[a]	223	97
Higher	76	569	395	1,589	183,912	39[b]	235	92
SEM	5	20	14	53	11,869	2	6	2
EST week 3								
Lower	69	566	389	1,572	176,244	47[a]	225	91
Control	77	568	418	1,684	193,905	39[b]	233	97
SEM	5	20	14	53	11,877	2	6	2
EST week 2 x week 3								
Control x Lower	65	561	381	1,552	167,496	49	207[b]	90[b]
Control x Control	76	569	444	1,782	204,980	43	239[a]	104[a]
Higher x Lower	74	571	398	1,592	184,993	45	242[a]	93[b]
Higher x Control	77	567	393	1,585	182,830	34	227[ab]	91[b]
SEM	7	28	20	74	16,751	3	9	3
P-value								
week 2	0.45	0.88	0.40	0.29	0.89	0.024	0.21	0.14
week 3	0.34	0.93	0.15	0.14	0.29	0.007	0.39	0.07
week 2 x week 3	0.59	0.84	0.10	0.12	0.25	0.44	0.016	0.016

[1]n=12, n=11, n=12, n=11 for treatments Control x Lower, Control x Control, Higher x Lower, Higher x Control, respectively.

[2] Averaged per 10 follicles. Cell density = the % of area within a follicle that is covered with cells.

[3] Averaged per 10 villi or crypt. n=13, n=12, n=11, n=12 for treatments Control x Lower, Control x Control, Higher x Lower, Higher x Control, respectively.

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

Jejunum

An interaction was found between EST week 2 and EST week 3 for both villi length and crypt depth (both P=0.016; Table 1). In the Control x Lower EST group villi length was approximately 16% shorter (Δ=32 µm; P=0.016) and crypt depth approximately 16% shallower (Δ=14 µm; P=0.016) compared to the other three EST treatment groups. Villi length or crypt depth did not differ between sexes (P≥0.19; data not shown). Histopathology score did not differ between treatments (P≥0.24, data not shown) or between sexes (P=0.86; data not shown).

H:L ratio

No interaction between EST week 2 and EST week 3 was found (P≥0.30) for the number of heterophils or lymphocytes or H:L ratio at hatch (Table 2). Higher EST in week 2 did not affect the number of heterophils or lymphocytes (P>0.11), but it tended to decrease the H:L ratio by 33 % compared to Control (Δ=2.1 ratio; P=0.07). Lower EST in week 3 resulted in 10% fewer lymphocytes (Δ=10 cells, P=0.01), 7 % more heterophils (Δ=9 cells, P=0.02), and a 38% higher H:L ratio (Δ=2.5 ratio, P=0.04) compared to Control. Levels and ratio of heterophil or lymphocytes did not differ between sexes (P≥0.30).

Table 2. Effect of a higher eggshell temperature (**EST**) of 38.9°C during week 2 of incubation (**Higher**) and/or a lower EST of 36.7°C during week 3 of incubation (**Lower**) compared to a control EST of 37.8°C (**Control**) on heterophil and lymphocyte occurrence in blood of broiler chicks at hatch.

Treatment	n	Lymphocytes (no./100 cells)	Heterophils (no./100 cells)	H:L ratio
EST week 2				
Control	14	16	81	6.4
Higher	11	22	76	4.3
SEM		2	3	0.8
EST week 3				
Lower	9	14[b]	83[a]	6.6[a]
Control	16	24[a]	74[b]	4.1[b]
SEM		2	3	0.8
EST week 2 x week 3				
Control x Lower	5	12	86	8.3
Control x Control	9	21	76	4.6
Higher x Lower	4	17	81	4.9
Higher x Control	7	27	71	3.6
SEM		3	4	1.1
P-value				
week 2		0.11	0.14	0.07
week 3		0.010	0.012	0.036
week 2 x week 3		0.86	0.96	0.30

[a-b]Least squares means within a column and factor lacking a common superscript differ (P≤0.05).
Abbreviation: H:L, heterophil to lymphocyte ratio.

NCD vaccination response

No interaction between EST week 2 and EST week 3 was found for NCD titer before vaccination (hatch), at 7 days post vaccination (day 22), and at 27 days post vaccination (Table 3; $P \geq 0.58$). Higher EST in week 2 had no effect on NCD titer before vaccination and at 7 days post vaccination ($P \geq 0.38$), but the 27 days post vaccination (at slaughter) NCD titer was higher compared to Control ($\Delta = 0.2$ log10titre; $P = 0.02$). Lower EST in week 3 had no effect on NCD titer before vaccination and at 7 days post vaccination ($P \geq 0.15$), but the 27 days post vaccination (at slaughter) NCD titer tended to be higher compared to Control ($\Delta = 0.1$ log10titre; $P = 0.08$). Females had higher NCD titers at slaughter age compared to males (2.9 vs 2.6 log10titer for females and males respectively; $P = 0.02$). At hatch and at 7 days post vaccination NCD titer did not differ between sexes ($P \geq 0.28$; data not shown).

Table 3. Effect of a higher eggshell temperature (**EST**) of 38.9°C during week 2 of incubation (**Higher**) and/or a lower EST of 36.7°C during week 3 of incubation (**Lower**) compared to a control EST of 37.8°C (**Control**) and sex on Newcastle disease (NCD) titer of broilers at hatch, at day 22 (7 days post NCD vaccination), and slaughter age (day 41 or 42).

	NCD titer		
Treatment	**Hatch[1] (log10titer)**	**Day 22[2] (log10titer)**	**Slaughter[2] (log10titer)**
EST week 2			
Control	3.2	1.5	2.6[b]
Higher	3.1	1.7	2.8[a]
SEM	0.1	0.1	0.1
EST week 3			
Lower	3.0	1.5	2.8
Control	3.2	1.7	2.7
SEM	0.1	0.1	0.1
EST week 2 x week 3			
Control x Lower	3.0	1.4	2.7
Control x Control	3.3	1.6	2.6
Higher x Lower	3.0	1.6	2.8
Higher x Control	3.1	1.7	2.8
SEM	0.1	0.2	0.1
P-value			
week 2	0.38	0.41	0.02
week 3	0.15	0.50	0.08
week 2 x week 3	0.58	0.79	0.82
sex	0.28	0.60	0.02

[1] n=13, 12, 12, 12 for treatments Control x Lower, Control x Control, Higher x Lower, Higher x Control respectively
[2] n = 16 / treatment

Table 4. Effect of a higher eggshell temperature (**EST**) of 38.9°C during week 2 of incubation (**Higher**) and/or a lower EST of 36.7°C during week 3 of incubation (**Lower**) compared to a control EST of 37.8°C (**Control**) and sex on natural antibodies (NAb) titer against keyhole limpet hemocyanin of broilers at hatch, day 14, 22, and slaughter age (day 41 or 42).

	NAb titer			
Treatment	**Hatch**[1]	**Day 14**[2]	**Day 22**[2]	**Slaughter**[2]
EST week 2				
Control	9.2	3.5	2.3	2.4
Higher	9.6	3.6	2.2	2.5
SEM	0.5	0.2	0.2	0.2
EST week 3				
Lower	9.0	3.4	2.2	2.4
Control	9.8	3.7	2.4	2.4
SEM	0.5	0.2	0.2	0.2
EST week 2 x week 3				
Control x Lower	8.7	3.4	2.3	2.4
Control x Control	9.7	3.6	2.4	2.3
Higher x Lower	9.3	3.5	2.2	2.5
Higher x Control	10.0	3.7	2.3	2.5
SEM	0.7	0.2	0.2	0.2
P-value				
week 2	0.54		0.74	
week 3	0.24		0.38	
week 2 x week 3	0.87		0.93	
sex	0.70		0.76	
week 2 x day	n.a.		0.80	
week 3 x day	n.a.		0.74	
week 2 x week 3 x day	n.a.		0.97	
day	n.a.		<0.001	
sex x day	n.a.		0.08	

[1] n=13, 12, 12, 12 chicks for treatments Control x Lower, Control x Control, Higher x Lower, Higher x Control respectively

[2] n=16 pens / treatment

Natural antibodies

NAb binding KLH at hatch, day 14, day 22, and slaughter age were neither affected by an interaction between EST in week 2 and EST in week 3 (Table 4; P=0.87) nor by a main effect of EST in week 2 or week 3 (P≥0.24). NAb titers were higher at day 14 compared to day 22 and at slaughter age (P<0.001), but did not differ between day 22 and slaughter age (titer was 3.5, 2.3, 2.4 for day 14, 22, slaughter respectively). Females tended to have higher NAb binding KLH

titer at day 22 (P=0.07) compared to males (2.5 vs 2.0 ± 0.2 respectively; data not shown), but NAb binding KLH titers did not differ between sexes at other ages (P≥0.68).

Mortality

Mortality rate was neither affected by an interaction between EST in week 2 and EST in week 3 (P=0.73; data not shown) nor by a main effect of EST in week 2 (P=0.73). Lower EST in week 3 tended to increase mortality rate (Δ=3.1%; P=0.10) compared to Control.

DISCUSSION

The aim of this study was to investigate whether a higher EST of 38.9°C in week 2 in combination with a lower EST of 36.7°C in week 3 of incubation affects primary immune organ development and post-hatch immune response in broilers compared to a constant EST of 37.8°C. Although no interaction was found between EST week 2 and EST week 3 for the majority of variables that were measured in the current study, some effects were found from either a higher EST in week 2 or a lower EST in week 3.

A higher EST of 38.9°C in week 2 tended to decrease the peripheral H:L ratio at hatch compared to a constant EST of 37.8°C. The H:L ratio has been shown to be associated with parasitic infection, because lymphocytes migrate to the site of infection (Davis et al., 2004; Lobato et al., 2005). However, in the current experiment any effect of an infection on the H:L ratio was not very likely, because the H:L ratio was detected in blood that was collected immediately after removing chicks from the incubator (within 12 hours after hatch). Moreover, a parasitic infection would also have caused an increase in monocyte and eosinophil numbers (Jain, 1986; Davis et al., 2004), but no eosinophils were found in any of the blood smears and only 2.2% of all leukocytes were monocytes (data not shown). The H:L ratio has also been used to indicate to what extent a chicken experienced physical or physiological stress (Gross and Siegel, 1983; Beuving et al., 1989; Altan et al., 2000; Davis et al., 2000; Elston et al., 2000; Onbaşilar and Aksoy, 2005; Nicol et al., 2006), because a stressor results in higher corticosterone levels and corticosterone lowers the number of peripheral lymphocytes and increases the number of heterophils (Shapiro and Schechtman, 1949; Weller and Schechtman, 1949; Bannister, 1951; Glick, 1958; Wolford and Ringer, 1962; Bishop et al., 1968; Dhabhar, 2002). Other studies found that thermal manipulations during incubation can cause heat or cold stress to the embryo (Givisiez et al., 2001; Moraes et al., 2002). However, it is not likely that the difference in EST during week 2 (E7 to E14) from the current experiment induced embryonic stress and caused the difference in H:L ratio at hatch moment. Stress alters corticosterone levels via the hypothalamic-pituitary-adrenal axis and this axis seems functional only from E13 or E14 onwards (Case, 1951, 1952; Woods et al., 1971; Scott et al., 1981; McIlhone, 2011). Also, heterophils from neonatal chicks are naïve and inefficient

(Zulkifli and Siegel, 1994; Lowry et al., 1997; Wells et al., 1998; Genovese et al., 2000) and might therefore be unreactive to corticosterone. Alternatively, the lower H:L ratio at hatch after a higher EST of 38.9°C in week 2 of incubation might be the result of a difference in proliferation of lymphocytes. Regardless of treatment group, the H:L ratio at hatch moment was high (≥3.6) in the current experiment. Other studies demonstrated that a high H:L ratio is normal in newly hatched chicks and that this ratio decreases during the first days of life (Lucas and Jamroz, 1961; Burton and Harrison, 1969; Zulkifli and Siegel, 1994, Maxwell et al., 1997; Gonzales et al., 2003; Oznurlu et al., 2010). The H:L ratio probably decreases during early life because lymphocytes start to migrate from maturation sites, such as the bursa, to the periphery during these days (Lucas and Jamroz, 1961; Cain et al., 1969; Jankovic et al., 1975; Lasilla, 1989). The lower H:L ratio in combination with a lower cell density within bursal follicles at hatch when EST in week 2 was 38.9°C could indicate that more lymphocytes proliferated already from the bursa to the peripheral blood. There are indications that a lower H:L ratio at hatch is predictive of improved later life production and reproduction traits (Al-Murrani et al., 2006) and, if this lower H:L ratio is consistent over time, of higher immune response against a Salmonella typhimurium infection (Al-Murrani et al., 2002). On the one hand, broilers in the current study incubated at a higher EST of 38.9°C in week 2 had higher NCD antibodies at slaughter and therefore the lower H:L ratio that was found at hatch in this group might indeed be predictive for a higher immune response in later life. On the other hand, a lower mortality rate and higher NAb titer would have strengthen the results further, but no effect was found of a higher EST of 38.9°C in week 2 on NAb titer or mortality rate. In layer type chicken, NAb levels binding to KLH have been found to negatively correlate with survival (Star et al., 2007; Sun et al., 2011; Wondmeneh et al., 2015) and mortality and morbidity levels after an avian pathogenic *Escheria coli* challenge (Berghof et al., 2019). However, immune response differs between broiler and layer type chicken (Koenen et al., 2002; Simon et al., 2016) and therefore, the correlation between NAb titre binding KLH and immunocompetence that has been shown in layers, might not be similar for broilers.

A lower EST of 36.7°C in week 3 was expected to improve embryo development compared to a constant EST of 37.8°C. Maatjens et al. (2016) showed that a lower EST of 36.7°C from E15 onwards resulted in chicks with a higher yolk-free body mass and a higher relative heart weight at hatch than an EST of 37.8°C. In the current experiment, no differences in yolk-free body mass were found when EST was lowered to 36.7°C in week 3 compared to a constant EST of 37.8°C (Wijnen et al., 2020). Moreover, the current study found some indications that the lower temperature in the last week of incubation impaired organ maturation instead of improving embryo development. For instance, a lower EST of 36.7°C in week 3 resulted in a higher cell density within bursal follicles and a lower number of peripheral lymphocytes in ratio to heterophils, which probably indicates impaired proliferation of B-cells from the bursa as discussed above. Also, jejunum villi length at hatch was shorter after a lower EST of 36.7°C in week 3 compared to a constant EST of 37.8°C. Intestinal villi grow rapidly during

the last 5 days before hatch (Uni et al., 2003; Uni et al., 1995; Grey, 1972). Lowering the EST during these last days of incubation in the current experiment might have slowed down villi growth. Chicken embryos act mainly as poikilotherms (French, 1997) and therefore a lower EST than 37.8°C could have slowed down embryo metabolism and consequently growth of organs such as the intestines. Studies evaluating the effect of a lower EST than 37.8°C during late incubation on intestinal morphology at hatch are lacking. Studies evaluating the opposite effect, an EST higher than 37.8°C during late incubation, did not find the opposite effect of longer intestinal villi at hatch (Barri et al., 2001). In fact, it seems that higher EST during late incubation impairs intestinal development instead (Wineland et al., 2006). However, the finding that a higher EST than 37.8°C during late incubation impairs intestinal villi length does not rule out the possibility that a lower EST than 37.8°C in this period slowed down intestinal villi growth. During late incubation the amount of oxygen available to the embryo is limited by the conductance of the eggshell (Visschedijk et al., 1985; Rahn et al., 1974; Romijn and Roos, 1938). Raising temperature during this period likely results in an imbalance between metabolic rate and oxygen availability and consequently in impaired organ development (Lourens et al., 2007), whilst lowering temperature during this period cannot cause this imbalance. Whether the lower EST of 36.7° during the last week of incubation has consequences for broiler immune response in later life remains unclear from the current results. After hatch, no significant differences in NCD titers and NAb titers were found between a lower EST of 36.7°C in week 3 and a constant EST of 37.8°C, suggesting that there were no differences in immune competence in later life. Nevertheless, a tendency (P=0.10) for a higher overall mortality in the 36.7°C EST treatment shows that overall broilers health might have been worse in this group. The possible difference in broiler mortality, which mainly occurred at the end of the grow-out period, might not be related to a difference in immune response or it might not be related to a difference in NCD vaccination response and NAb titers at the time points in the current study, but more related to other physiological differences such as thermoregulatory competences.

To predict effects of a higher EST of 38.9°C in week 2 or a lower EST of 36.7°C in week 3 on later life immune response more research is suggested in challenging environments. The current experiment was conducted in a research facility with accurate and optimal management procedures and at low animal stocking densities. It is likely that the immune system was therefore only activated to a relatively low level. Any possible difference in functioning of the immune system might not have been revealed or detected. A follow up study in which the response of broilers to a validated challenge model can be measured accurately at very specific time points might reveal whether incubation temperature patterns have beneficial or detrimental effects for later life broiler immune response or whether it does not make a difference compared to a constant EST of 37.8°C.

In conclusion, immune organ development at hatch and immune response in later life does not seem to be influenced by the incubation temperature pattern with a higher EST of

38.9°C in week 2 and a lower EST of 36.7°C in week 3. Nevertheless, a higher EST of 38.9°C in week 2 as well as a lower EST of 36.7°C in week 3 both affected immune organ development at hatch and immune response post hatch compared to a constant EST of 37.8°C. A higher EST of 38.9°C in week 2 showed some indications that immune organ development and immune response were perhaps positively affected, whilst a lower EST of 36.7°C in week 3 showed some indications that immune organ development and immune response were negatively affected. Yet, only some immune parameters were studied and only during fixed time points, so more research is needed to confirm or disprove these indication and to study whether incubation temperature patterns affect primary immune organ development and post-hatch broiler immune response.

ACKNOWLEDGMENTS

The authors wish to thank HatchTech for funding this project, Lagerwey hatchery for providing the eggs, BSc student Lianne Cheung for her histological work on the Bursa of Fabricius, and the animal caretakers from Carus, MSc student Lara olde Bolhaar, WUR colleagues (Bjorge Laurenssen, Marcel Heetkamp, Ilona van den Anker-Hensen, Monique Ooms, Henny Reimert, Bahadir Can Güz, and Joop Arts), and HatchTech colleagues (Marieke Priester and Gerald Aalbers) for their assistance.

Conflict of Interest Statement: Authors H. J. Wijnen, I. A. M. van Roovert-Reijrink, and C. W. van der Pol are employed by company HatchTech. All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 4

EFFECTS OF LATE INCUBATION TEMPERATURE AND MOMENT OF FIRST POST-HATCH FEED ACCESS ON NEONATAL BROILER DEVELOPMENT, TEMPERATURE PREFERENCE, AND STRESS RESPONSE.

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Under review at Poultry Science

ABSTRACT

Early life experiences are known to be of great importance for later life. For instance, stress during early life can increase fearfulness at later age. In broilers, delayed feeding after hatch may cause stress. Besides, delayed feeding after hatch may affect neonatal broiler development and thermogenesis and consequently preferred ambient temperature. Moreover, these effects of feeding strategy may be dependent on late incubation temperature. Eggs from a 54 wk old broiler breeder flock were incubated at 37.8°C (**control**) or 36.7°C (**lower**) eggshell temperature (**EST**) during late incubation (≥ embryonic d 17). At hatch, two feeding strategies were applied (direct access (**early feeding**) or 51-54 h delayed access (**delayed feeding**)). Broilers were equally divided over 32 pens and grown for 3 wk. Stress was assessed by corticosterone in blood at 0h, 48h, 96h and d21 after hatch. Fearfulness was assessed by tonic immobility at d13. Temperature preference was assessed at d2 and d12. Broiler development was determined at 0h, 48h, and 96h after hatch. There was no interaction EST x feeding strategy for any parameter ($P \geq 0.07$). Early feeding resulted in 2.5x lower plasma corticosterone concentration at 48h ($P < 0.01$) and a 2.2°C and 2.0°C lower preference temperature for d2 and d12 respectively ($P = 0.01$) compared to delayed feeding. Tonic immobility was not affected. In conclusion, early feeding reduces stress on the short term and stimulates thermoregulatory ability of broilers on the longer term.

INTRODUCTION

The perinatal period of animals is known to be of great importance for their later health, welfare, and performance. This period in life contains critical windows during which the phenotype can be shaped permanently by environmental factors. Such alterations in phenotype during early life can determine animal behaviour during environmental challenges in later life. For example, in laying hens it was shown that injection of corticosterone in eggs, as a model for prenatal stress, resulted in higher fear responses of hens at 2 wk post hatch (Janckzak et al., 2006).

One event during the perinatal life of poultry that may cause stress is delayed access to feed and water after hatch moment (referred to as "**delayed feeding**"). For practical reasons, most chicks in commercial practice are withheld from feed and water during the period from hatch at the hatchery until placement at the farm. Withholding or partial restriction of feed and/or water for 4 h or longer has been shown to cause a stress reaction in chickens aged 3 weeks or older, indicated by higher plasma corticosterone concentrations, increased peripheral heterophil to lymphocyte ratio, and/or behavioural changes (Buckland et al., 1974; Nir et al., 1975; Freeman et al., 1980; Scott et al., 1983; Hocking et al., 1996; Najafi et al., 2015; Pál et al., 2015). However, studies that investigated whether delayed feeding causes stress in neonatal chicks are limited (Gonzales et al., 2003; van de Ven et al.,2013; Khosravinia & Manafi, 2016; Shakeel et al., 2016) and their results are inconsistent (reviewed by de Jong et al., 2017). Moreover, possible effects of neonatal stress due to delayed feeding on stress response at later age are unknown.

Besides trough induction of stress, delayed feeding may evoke it's effects through other physiological mechanisms. For instance, it was shown that providing feed and water directly after hatch (referred to as '**early feeding**'; Roberts et al., 1928) increased metabolic heat production and improved resistance of neonatal chicks against cold exposure (Decuypere & Kuhn, 1988; van den Brand et al., 2010; van Roovert-Reijrink et al., 2017). This may suggest that early fed broilers may prefer a lower ambient temperature compared to delayed fed broilers during grow out, but this has not been studied yet.

An effect of early or delayed feeding strategy after hatch on preferred ambient temperature during rearing might depend on incubation temperature. It has been shown that temperature during late incubation can affect preferred ambient temperature post hatch. For example, a higher incubation temperature from day 13 of incubation onwards resulted in a ~ 1.5°C higher preferred ambient temperature up to 15 days post hatch (Morita et al., 2016a, 2016b). A lower incubation temperature during the last week of incubation tended to result also in a 1.4°C higher preferred ambient temperature at d 1 post hatch (Wijnen et al., 2020). Thus, early fed chickens may prefer a lower ambient temperature during rearing compared to delayed fed chickens when late incubation temperature was standard, whereas this may not be true at higher or lower late incubation temperatures.

De Jong et al. (2017) suggested that effects of early feeding may indeed be dependent on incubation temperature. They showed that early feeding can affect mortality rate and neonatal body development in terms of organ weights, gut development, immune response, and RY uptake. However, considerable variation and inconsistency among studies existed and these ambiguous results might be related to, amongst other factors, incubation temperature. Incubation temperature is known to have a major impact on embryo development and consequently chick quality at hatch. So far, a constant eggshell temperature (**EST**) of 37.8°C throughout incubation is regarded optimal (Lourens et al., 2005). However, a lower EST of 36.7°C during the last week of incubation was shown to increase yolk free body mass (**YFBM**) and heart weight at hatch (Maatjens et al., 2016; Wijnen et al., 2020). Newborn chicks with a higher YFBM have relatively less RY available that could be used for body development during the neonatal period. Therefore, the importance of early feed intake may be higher for these chicks.

We hypothesize that delayed feeding would be stressful for the neonatal chick, resulting in higher fearfulness at later age. Additionally, delayed feeding might result in higher preferred ambient temperatures during rearing. Finally, late incubation temperature might interact with post-hatch feeding strategy on temperature preference and neonatal chick development.

MATERIAL AND METHODS

An experiment was set up as a 2 x 2 factorial arrangement with two EST's during late incubation and two feeding strategies after hatch. EST from embryonic d (**E**) 17 onwards was set either at 37.8°C (**control**) or at 36.7°C (**lower**), whereas feeding strategy was either access to feed and water within 3 to 6h after hatch (**early feeding**) or within 51 to 54 h after hatch (**delayed feeding**). The experimental protocol was approved by the Governmental Commission on Animal Experiment, The Hague, the Netherlands, approval number: 2018.W-0020.001 and by the Ethical Committee of Poulpharm, Belgium, approval number P19034-FP. A brief description of material and methods is provided in this chapter. For more details please see Wijnen et al. (2021).

Incubation

In total 1,338 eggs from a 54-wk-old Ross 308 broiler breeder flock were divided over 16 setter trays (type 88 Setter Tray, HatchTech, Veenendaal, the Netherlands). All 16 trays were set in one climate respiration chamber, which was used as incubator (details provided by Heetkamp et al., 2015) in which 4 EST sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK) were attached to individual eggs. Incubator temperature was continuously adjusted, based on the median temperature of these 4 EST sensors to aim at an EST of 37.8°C

up to E17. RH was maintained between 50 and 55%, CO_2 level was maintained below 3,500 ppm, and eggs were turned every h by an angle of 45° from horizontal.

At E17, all eggs were candled and eggs containing a viable embryo (86.7% of fertile eggs at set) were transferred to one hatching basket per setter tray. Hatching baskets were divided over 4 incubators in which EST control was performed as described above. Two incubators were set at an EST of 37.8°C, whereas the other 2 incubators were set at an EST of 36.7°C. RH was maintained between 45 and 75% and CO_2 levels were maintained below 3,500 ppm. At 468 h after the onset of incubation (E19 12h), the air temperatures in the incubators were fixed at their actual temperature setting and EST was allowed to change.

Hatch and Early Feeding

From 468 h of incubation onward, the incubators were opened every 3 h to check whether or not chicks had hatched. Any newly hatched chick was marked, put back in the original hatching basket in the incubator and left to dry for 3 h. After 3 h, the chick was pulled from the incubator and classified either as 1st grade chick (no abnormalities) or 2nd grade chick if any abnormality was observed (e.g. crossed beak, blindness, exposed brains, extra legs, exposed yolk). Any 1st grade chick (n=1,028) was feather sexed, received a unique neck tag, and was transferred to a HatchCare basket (HatchTech, Veenendaal, the Netherlands) that was placed in a chick storage room at 36.0°C and 55% RH until 516 h after the onset of incubation (E21 12 h). In the chick storage room, half of the baskets were provided *ad libitum* with fresh water and starter pellet feed (early feeding; diet details given in 'grow out' section below) whereas no feed and water was provided in the other half of the baskets (delayed feeding). At 516 h after the onset of incubation, all chicks were transported in a climate-controlled van (29.4°C and 36% RH) during approximately 3 h to a grow out facility in Zwevegem, Belgium.

Grow-out

Housing. Chicks were divided over 32 floor pens in one broiler house with 30 broilers (15 male and 15 female) per pen and grown for 3 wk. Pens were divided over 8 equal blocks and each block contained all 4 treatment groups. Pen size was 260 x 105 cm. The floor was covered with wood shavings and within each pen 4 drinking nipples and a feed silo were provided. House temperature setpoint was 35°C at placement and was linearly decreased to 22.5°C at d 21. A heating lamp was provided in the middle of each pen from placement until d 12, meaning that broilers were able to choose their own preferred ambient temperature. RH was on average 34.5% ± 15%. Continuous light was provided until d 2 and thereafter 1 h of darkness / 24h was added each d until 18L:6D was provided by d 7 and onwards.

Diet and vaccinations. Pens from the delayed feeding treatment were divided with a fence in the middle into an unfed side and a fed side. The litter of the unfed side was covered with cardboard to prevent litter consumption. At placement, all delayed fed broilers were positioned in the unfed side of the pen and each broiler was relocated individually to the fed

side of the pen 48 h after it had received its neck tag. Fences and cardboard were removed after all broilers were relocated to the fed side of the pen.

A starter pelleted diet was provided from hatch until d 13 and a grower pelleted diet from d 13 until d 21. Both diets did not contain coccidiostats and were provided ad libitum. The grower diet included fish meal (10%) and rye (5%) as predisposing factors for the development of necrotic enteritis after d 21 (see Wijnen et al., 2021). Newcastle disease (Avishield ND) and infectious bronchitis (Poulvac IB primer) spray vaccinations were administered at placement. At d 12, Newcastle disease (Avishield ND) and Gumboro disease (Nobilis Gumboro D78) vaccinations were provided via drinking water.

Data collection

Eggs were weighed at set and at E17, prior to the onset of EST treatments, to calculate egg weight loss. Eggs candled out at E17 were opened and scored either as infertile or as dead embryo. Actual EST from all eggshell sensors was logged every minute. For each sensor, average EST per h was calculated. These averages were used to calculate average, minimum, and maximum EST per h per treatment. Effects of EST on incubation and embryo development were studied by determining hatchability, hatch window, incubation duration, and chick quality at hatch. Hatchability was calculated per hatching basket as the number of hatched chicks divided by the number of eggs that contained a viable embryo at E17. Incubation duration was calculated as the number of h from E0 (start of incubation – excl. warming profile) to emergence from the eggshell. Hatch window was calculated as incubation duration of the last chick minus incubation duration of the first chick.

Chick quality of 1st grade chicks was determined by measuring BW, chick length, and navel score as described by Wijnen et al. (2020). Additionally, every twelfth chick per treatment that hatched was euthanized through decapitation until 25 chicks per treatment were collected. These chicks were opened and residual yolk (**RY**), heart, intestines, bursa, and stomach were removed and weighed on a three-decimal scale. Stomach included the proventriculus, the intermediate zone, the ventriculus, and the pylorus. YFBM was calculated as BW minus RY weight. Relative organ weights were calculated as percentage of YFBM. After weighing the intestines, approximately 1.5 cm of the jejunum was collected (0.5 cm posterior the distal part of the duodenum). A Swiss roll was made as described by Moolenbeek and Ruitenberg (1981), fixated in 4% formaldehyde in PBS for 2 d, and stored in 70% ethanol until processing. At processing, tissues were embedded in paraffin, 3 µm sliced, mounted on a glass microscope slide, and hematoxylin and eosin stained. From 12 random chicks per treatment, one slide was analyzed on a microscope (Leica DM3000 LED, LAS V4.9-software) for villi and crypt appearance. From each slide, 5 to 10 villi and associated crypts were randomly chosen. Villi lengths and crypt depths were determined as described by Uni et al. (1995) and the averages of these 5-10 villi and crypts per slide were calculated and used for statistical

analysis. Villi : crypt ratio (**V:C**) was calculated as villi length divided by crypt depth for each individual villi and associated crypt, whereafter the averages were used for statistical analysis.

After decapitation, blood was collected in serum tubes (Vacuette 5 ml, Greiner Bio-One), stored on ice, and serum was collected after centrifugation at 5,251 x g for 10 min at 4°C. Serum was stored at -20°C until samples were analyzed for natural antibodies against keyhole limpet hemocyanin (**NAb**) isotypes IgM and IgY according to an adjusted protocol from Lammers et al. (2004), described by Wijnen et al. (2021). Titers were calculated according to the protocol from Van der Klein et al. (2015).

Neonatal broiler development was determined at 48 and 96 h after hatch. At both ages, 1 female and male broiler were selected randomly per pen, weighed, and euthanized by decapitation. RY, YFBM, relative bursa and heart weight, villi and crypt morphology, and IgY and IgM NAb were determined as described in the previous paragraph. NAb IgM isotype was not found in serum of any broiler and will therefore not be discussed any further. Mortality and culled broilers (humane end points as defined by Marchewka et al., 2013) were recorded daily during the 1st wk. Mortality percentage was calculated per pen by summing up the number of broilers that died and were culled as percentage of broilers at placement minus the number of broilers that were dissected.

Temperature preference was determined at d 2 (4 pens per treatment group) and d 12 (5 pens per treatment group) by observing behavior during a temperature preference test that was adapted from the protocol of Walstra et al. (2010). Two male and 2 female broilers were selected randomly per pen and were placed together in the middle of a test box which was situated in a room adjacent to the broiler house. This wooden box (160 x 60 x 50 cm) was covered with a Plexiglas lid, had wood shavings at the bottom, and 2 infrared heat bulbs (250 Watt each) situated at one side of the box such that a linear temperature gradient from 20°C to 50°C was created. Temperature sensors at broiler height (NTC DC95 thermistors, Thermometrics, Somerset, UK) continuously monitored the actual temperature at 24 locations in the box equally spread over the total gradient. Generally, broilers laid down and remained on the same position from approximately 15 min after placement onwards. Therefore, 20 min after placement in the box, the location of each broiler was noted as well as the associated actual sensor temperature. From d 1 to d 7, in all pens cloacal temperature (VT1831, Microlife, Widnau, Switzerland) was determined each morning in the same 3 broilers per pen (at least 1 broiler of both sexes was included).

Stress response was studied by measuring corticosterone, and tonic immobility. Corticosterone was analyzed in serum of all broilers that were dissected at hatch, 48 h after hatch, and 96 h after hatch. Additionally, corticosterone was analyzed in serum collected at d 21 from in total 100 broilers (details provided in Wijnen et al., 2021). Corticosterone was analyzed in duplo in all serum samples. Serum was diluted 5x with steroid diluent from a radioimmunoassay kit (RIA A68390, Beckman Coulter, Indianapolis, USA) and subsequently analyzed with this kit according to the protocol from Van der Pol et al. (2019).

Tonic immobility was assessed at d 13. In each pen, 1 male and female broiler were selected randomly and the latency until the broiler was attempting to stand up as well as the number of vocalizations and the number of attempts to put a broiler on the back were assessed according to a protocol adapted from Jones et al. (1986). A broiler was transported by hand from its home pen to a table in the corner of the broiler house. Two observers were standing next to the table without making eye contact with the broiler at any time during the test. One and the same observer induced tonic immobility by gently laying the broiler on its back on the table and consequently holding one hand on the sternum and lightly covering the eyes with the other hand during 10 s. After 10 s, the observer slowly removed both hands and meanwhile the other observer started recording. If the broiler stood up within 10 s after removing hands, the procedure was repeated up to a maximum of 5 times. After 5 unsuccessful attempts, the test was stopped, and another pen mate was selected randomly. Latency until attempt to stand up was recorded once a broiler remained down for at least 10 s after removing hands. If a broiler did not stand up within 600 s, the test was stopped and a latency of 600 s was noted.

Statistical analyses

Data was analysed with the statistical software package SAS (Version 9.4, SAS institute, 2010). The basic model used for all data at hatch was

$$Y_i = \mu + EST_i + e_i, \quad [1]$$

where, Y_i = the dependent variable, μ = the overall mean, EST_i = eggshell temperature during late incubation ($_i$ = 36.7°C or 37.8°C), and e_{ij} = the error term. To analyse hatchability, hatching basket was considered to be the experimental unit and incubator was added as a random factor to model 1. All other data at hatch was collected for individual chicks, but hatching basket was considered to be the experimental unit by extending model 1 with hatching basket nested within incubator as a random factor. Sex was added to model 1 as a fixed factor. Preliminary statistical analysis did not show significant effects of EST x sex for any of the variables, except for navel score. Therefore, interactions between sex and treatments were excluded from the final model with the exception of navel score. For chick length, the ID code of the person that performed the measurement was added to the model as a fixed factor.

To analyse data that was collected after hatch, model 1 was extended with feeding strategy and sex as follows;

$$Y_{ij} = \mu + EST_i + FEED_j + sex_k + \text{interactions} + e_{ijk}, \quad [2]$$

where $FEED_j$ = feeding strategy ($_j$ = early or delayed), sex_k = sex (k = male or female), interactions = 2 and 3-way interactions between EST, FEED, and sex, and e_{ijk} = the error term. Preliminary statistical analysis did not show significant effects of 3-way interactions,

sex x EST, or sex x FEED for any of the variables. Therefore, these interactions were excluded from the final model. All data after hatch was collected for individual broilers, but pen was considered to be the experimental unit by extending model 2 with pen as a random factor with the exception of mortality. Mortality was collected per pen and therefore no random factor was added and sex was excluded from model 2.

The PROC MIXED procedure was used to analyse all data, except for navel score, number of vocalisations, and number of back attempts during tonic immobility. Model assumptions were verified by inspection of residual plots and not normally distributed data were log transformed. Data is expressed as least square mean ± SEM. Tukey adjustments for multiple comparisons were used to compare least square means. A P-value ≤0.05 was considered to be significant and a P-value >0.05 and ≤ 0.10 as a tendency.

The PROC GLIMMIX procedure was used to analyse navel score, number of vocalisations, and number of back attempts during tonic immobility. For navel quality score navel scores 2 and 3 were grouped and analysed as binary data, using a logit link function in model 1. Number of vocalisations and back attempts during tonic immobility were analysed with a Poisson log link function in model 2. Data is expressed as mean ± SE. A P-value ≤0.05 was considered to be significant and a P-value >0.05 and ≤ 0.10 as a tendency.

RESULTS

Incubation

Egg weight loss between set and E17 was on average 8.6%. Infertility and embryonic mortality of set eggs were 4.6% and 1.8%, respectively. Average, minimum, and maximum EST per h per treatment are provided as supplementary data (Figure S1). EST treatment had no effect on hatchability (P=0.26; average 97.2% ± 1.2) nor on duration of the hatch window (37 h for both EST treatments). Incubation duration was on average 7 h longer for lower EST compared to control EST (P<0.001; Table 1).

Chick quality at hatch

Lower EST resulted in a 1 mm shorter chick length (P<0.001) and 9.7% higher relative heart weight (P=0.04) compared to control EST (Table 1). Navel score showed an interaction between EST and sex (P<0.001; data not shown). At lower EST, female chicks had worse navel quality with approximately 17.5% higher incidence of navel score 2 or 3 (72.9%) compared to lower EST male chicks and control EST male and female chicks, which were equal to each other (53.6%, 57.5%, 55.3% score 2+3, respectively). No effect of EST was found for BW, RY, YFBM, NAb IgY, jejunum villus height or crypt depth or V:C, and relative weight of bursa, stomach, or intestines (P≥0.12; Table 1).

Table 1. Effect of eggshell temperature (**EST**) during late incubation (≥ embryonic d 17; 37.8°C (**control**) or 36.7°C (**lower**)) on chick quality characteristics at hatch (LSmean ±SEM).

n[1]	Duration (h)	BW(g)	RY[2] (g)	YFBM[2] (g)	Length (cm)	Heart (%)[3]	Bursa (%)[3]	NAb[2] (titre)	Stomach[2] (%)[3]	Intestines (%)[3]	Villus (µm)	Crypt (µm)	V:C[2] (ratio)
EST													
Control	479[b]	52.2	8.6	43.0	19.4[a]	0.72[b]	0.14	3.2	5.54	4.01	293	69	4.5
Lower	486[a]	52.0	8.2	43.2	19.3[b]	0.79[a]	0.13	3.3	5.45	4.07	328	73	4.5
SEM	1.1	0.08	0.23	0.27	0.07	0.024	0.007	0.25	0.139	0.114	21.3	5.1	0.21
P-values													
EST	<0.001	0.12	0.30	0.55	<0.001	0.04	0.60	0.83	0.63	0.73	0.30	0.58	0.97

[a-b] LSMeans within a column and factor lacking a common superscript differ (P<0.05).

[1] 8 hatching baskets / treatment (Duration, BW, length: 65 chicks / hatching basket, remaining characteristics: 25 chicks / treatment).

[2] RY= residual yolk, YFBM= yolk-free body mass, NAb= IgY natural antibody, stomach = proventriculus + ventriculus, V:C = jejunum villus : crypt ratio.

[3] Weight relative to YFBM.

Stress

No interaction between EST and feeding strategy was found for corticosterone at any age (P≥0.17; Table 2). Lower EST resulted in a 6.1 ng / ml higher corticosterone level at hatch (P=0.04) compared to control EST, whereas EST had no effect on corticosterone level at any other age (P≥0.44). Early feeding resulted in a 22.3 ng / ml lower corticosterone level at 48 h after hatch (P<0.0001) compared to delayed feeding, whereas feeding strategy had no effect on corticosterone level at any other age (P≥0.66).

No interaction between EST and feeding strategy or a main effect of EST or feeding strategy was found for any tonic immobility responses (P≥0.09; Table 3).

Table 2. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic d 17; 37.8°C (**control**) or 36.7°C (**lower**)) and/or feeding strategy after hatch (direct access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on blood corticosterone of broilers at hatch and 48 h, 96 h, and 21 d of age (LSmean ± SEM).

	Corticosterone (ng / ml)[1]			
	Hatch[1]	**48 h[2]**	**96 h[2]**	**d 21[3]**
EST				
Control	21.6[b]	25.0	15.9	6.0
Lower	27.7[a]	27.5	16.5	6.4
SEM	1.94	2.25	2.40	1.31
Feeding strategy				
Delayed	-	37.3[a]	16.2	6.6
Early	-	15.2[b]	16.2	5.8
SEM	-	2.28	2.41	1.31
EST x Feeding strategy				
Control x Delayed	-	35.3	13.4	5.4
Control x Early	-	14.8	18.3	6.6
Lower x Delayed	-	39.4	18.9	7.8
Lower x Early	-	15.6	14.1	5.0
SEM	-	3.19	3.39	1.83
P-values				
EST	0.04	0.44	0.85	0.81
Feeding strategy	-	<0.0001	0.99	0.66
EST x Feeding strategy	-	0.61	0.17	0.30

[a-b] LSMeans within a column and factor lacking a common superscript differ (P<0.05).
[1] n = 25 chicks / treatment (divided over 8 hatching baskets / treatment).
[2] n = 8 pens / treatment group (2 broilers / pen).
[3] n = 8 pens / treatment group (total 100 broilers, unequal nr per pen).

Table 3. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic d 17; 37.8°C (**control**) or 36.7°C (**lower**)) and/or feeding strategy after hatch (direct access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on the number of attempts or vocalizations (mean±SE) and latency to attempt to stand up (LSmean ±SEM) of broilers during a tonic immobility test at d 13.

	n[1]	Attempts (no.)	Vocalizations (no.)	Latency (sec)
EST				
Control	16	3 ±0.3	5 ±2.0	139
Lower	16	2 ±0.2	6 ±1.9	170
SEM				27.4
Feeding strategy				
Delayed	16	2 ±0.2	6 ±2.1	161
Early	16	3 ±0.3	5 ±1.8	148
SEM				27.4
EST x Feeding strategy				
Control x Delayed	8	2 ±0.4	7 ±2.7	125
Control x Early	8	3 ±0.5	4 ±2.9	154
Lower x Delayed	8	3 ±0.3	5 ±3.2	202
Lower x Early	8	2 ±0.2	6 ±2.1	143
SEM				38.5
P-values				
EST		0.28	0.72	0.62
Feeding strategy		0.69	0.78	0.96
EST x Feeding strategy		0.15	0.09	0.99

[1] Pens, with 1 male and 1 female broiler / pen.

Temperature preference

No interaction between EST and feeding strategy or a main effect of EST was found for preferred ambient temperature at d 2 or d 12 (P≥0.32; Table 4). Early feeding resulted in a 2.2°C and 2.0°C lower preferred ambient temperature at d 2 and d 12, respectively (P=0.01 for both d) compared to delayed feeding.

Cloacal temperature linearly increased from d 1 to d 7 (average 40.4 to 41.3 ±0.05 °C) and was neither affected by an interaction between EST and feeding strategy (P≥0.17; data not shown) nor by a main effect of EST (P≥0.12) or feeding strategy (P≥0.05).

Table 4. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic d 17; 37.8°C (**control**) or 36.7°C (**lower**)) and/or feeding strategy after hatch (direct access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on preferred ambient temperature of broilers at d 2 or d 12 of age (LSmean ±SEM).

	n[1]	Preferred ambient temperature (°C)	
		d 2	d 12
EST			
Control	8/10	28.3	25.2
Lower	8/10	28.2	25.9
SEM		0.55	0.52
Feeding strategy			
Delayed	8/10	29.4[a]	26.6[a]
Early	8/10	27.2[b]	24.6[b]
SEM		0.55	0.53
EST x Feeding strategy			
Control x Delayed	4/5	29.2	25.9
Control x Early	4/5	27.4	24.5
Lower x Delayed	4/5	29.5	27.3
Lower x Early	4/5	26.9	24.6
SEM		0.78	0.74
P-values			
EST		0.85	0.32
Feeding strategy		0.01	0.01
EST x Feeding strategy		0.64	0.40

[a-b] LSMeans within a column and factor lacking a common superscript differ (P<0.05).
[1] Pens at d 2 / d 12, with 2 male and 2 female broilers / pen.

Neonatal Development

No interaction between EST and feeding strategy was found for 1st week mortality (P=0.07), nor for any of the parameters assessed at 48 h after hatch (P≥0.05) or for any of the parameters assessed at 96 h after hatch (P≥0.28).

Lower EST had 5.9% higher 1st wk mortality compared to control EST (P<0.001; Figure 1). At 48 h after hatch, lower EST compared to control EST had higher jejunum V:C (P=0.04; ratio 4.2 vs. 3.9 ±0.11) and higher relative heart weight (P=0.01; 0.92 v 0.85 % ±0.017), whereas at 96 h after hatch these differences were not present anymore (P≥0.18). EST had no effect on BW, RY, YFBM, relative bursa weight, NAb titre, and jejunum villi length or crypt depth at 48 h or 96 h after hatch (P≥0.06; data not shown).

Feeding strategies did not differ in 1st wk mortality (P=0.12; Figure 1). At 48 h and at 96 h after hatch, early feeding compared to delayed feeding had higher BW (48 h: P<0.001; 58.3 vs. 44.4 g ± 0.61/ 96 h: P=0.02; 89.8 vs. 79.5 g ± 2.98), higher YFBM (48 h: P<0.001; 56.1

vs. 42.1 g ± 0.71 / 96 h: P=0.01; 90.0 vs. 78.6 g ± 3.02), and longer jejunum villi length (48 h: P<0.01; 420 vs. 375 µm ± 8.8 / 96 h: P=0.01; 497 vs. 429 µm ± 16.8). At 48 h after hatch, early feeding compared to delayed feeding had deeper jejunum crypt (P<0.001; 109 vs. 91 µm ± 2.3) and lower IgY NAb titre (P=0.04; titre 3.8 vs. 4.4 ± 1.7), whereas at 96 h after hatch these differences were not present anymore (P≥0.44). Feeding strategy had no effect on RY, relative heart weight, jejunum V:C, or relative bursa weight at 48 h or 96 h after hatch (P≥0.06; data not shown).

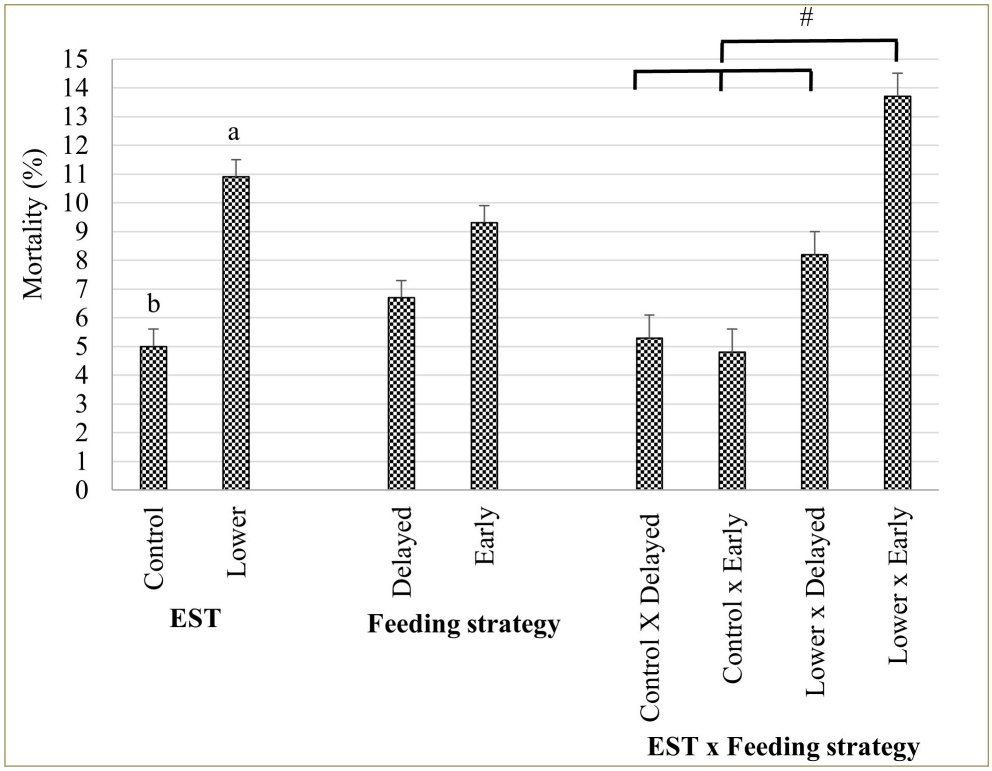

Figure 1. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic d 17; 37.8°C (**control**) or 36.7°C (lower)) and/or feeding strategy after hatch (direct access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on 1st wk mortality of broilers.
a-b LSmeans within a factor lacking a common superscript differ (P<0.05).
LSmeans tend to differ (P<0.10).
Error bars indicate SEM. n=8 pens / treatment group (30 broilers / pen).

DISCUSSION

We hypothesized that delayed access to feed and water after hatch would be stressful for chicks, resulting in higher fearfulness at later age. Furthermore, delayed feeding was expected to result in higher preferred ambient temperatures during rearing and a low EST during late incubation was suggested to interact with post-hatch feeding strategy on temperature preference as well as neonatal broiler development in general. Firstly effects of feeding strategy will be discussed. Subsequently, whether or not incubation temperature interacts with feeding strategy and finally, main effects of incubation temperature.

Feeding strategy

At 48 h after hatch, 2.5 times higher corticosterone levels were found in 48 h delayed fed broilers compared to early fed broilers. The higher corticosterone level found in delayed fed broilers was likely the result of stress due to feed and water deprivation. Corticosterone level increased between hatch and 48 h after hatch in delayed fed broilers, whereas in early fed broilers it decreased during that time span. Moreover, once delayed fed broilers had access to feed and water for 48 h, their corticosterone level reduced to similar level as that of early fed broilers. Our finding is in line with Van de Ven et al. (2013) who studied chicks that hatched either in a conventional hatcher without feed and water or in a Patio hatching system with direct access to feed and water. In a conventional hatcher, chicks that hatched early within the hatch window tended to have higher corticosterone levels at pull compared to chicks that hatched late during the hatch window, also shown by Tong et al. (2015), whereas in a Patio hatching system this difference between early and late hatchers was not present.

The increase in corticosterone in delayed fed broilers could also be the response to a lack of glucose. Delayed fed broilers likely had limited glucose available during the first 48 h after hatch, since most glycogen storages are used during hatching (Freeman, 1965; Christensen et al., 2001; Molenaar et al., 2013; van de Ven et al., 2013; Maatjens et al., 2014) and no exogenous glucose via the diet was provided. Blood glucose was not determined in the current study, but it has previously been shown that 48 h delayed fed broilers had lower blood glucose concentration after hatch compared to early fed conspecifics (Gaglo-Disse et al., 2010; Wang et al., 2014; Khosravinia & Manafi, 2016). Corticosterone level may be increased because corticosterone increases gluconeogenesis via decreasing uptake of glucose by muscle cells and increase of protein degradation to supply glucogenic amino acids (Mench, 2002). Consequently, in delayed fed broilers, corticosterone levels may rise, to maintain glucose homeostasis. However, it is unclear whether or not this mechanism occurs independent from stress or that it is also part of the stress response. In stressful situations there is a higher demand for direct accessible energy, such as glucose, for a fight or flee response. Furthermore, blood glucose concentration can remain lower during 2 wk post hatch after delayed feeding (Gaglo-Disse et al., 2010), whereas corticosterone level does not. Studies on early feeding

that measured additional stress indicators next to corticosterone are rare. Khosravinia and Manafi (2016) found that unfed day old chicks showed decreasing resting behaviour and increasing active wakefulness and attempt to escape from a box without feed and water as time progressed. Aforementioned findings suggest that the higher corticosterone that was found in the current study at 48 h after hatch in delayed fed broilers appears to be a stress response rather than an effect of a low blood glucose level.

We hypothesized that stress during early life due to delayed feeding would result in higher fearfulness at later age. In the current study, no indications were found that early and delayed fed broilers differ in fearfulness as no difference was found during a tonic immobility test at d 13. Besides, corticosterone level at d 21 was similar between both feeding strategies. It should be noted that corticosterone at d 21 was determined without applying a stressor. Hedlund and Jensen (2019) showed in laying hens that although chicks experienced more stress during hatchery processes, their baseline corticosterone levels at d 6 or 41 did not differ. Once these hens were manual restrained, corticosterone levels increased to a larger extent in hens that experienced more stress during early life, but without showing more fearful behaviour during a tonic immobility test. This suggests that feeding strategy during early life possibly affects stress responsiveness during later life when exposed to certain stressful conditions, even though baseline corticosterone level and behavioural response to a tonic immobility test did not differ in the current study.

Feeding strategy evidently affected preferred ambient temperature during rearing. Feeding increases thermogenesis by digestion of feed and body growth and early fed broilers produced approximately twice as much heat at pulling compared to delayed fed broilers (van Roovert-Reijrink et al., 2017). In the current study, early fed broilers had higher BW, YFBM, and longer jejunum villi length compared to delayed fed broilers up to at least 96 h after hatch. This supports the idea that early fed broilers are ahead in body growth and development and it suggests that thermogenesis will indeed be higher in early fed broilers. On the one hand, higher thermogenesis seems to beneficially affect cold tolerance. Early fed broilers showed milder drops in rectal temperature after cold exposure (20°C) at d 2-3 compared to delayed fed broilers (Van de Brand et al., 2010). On the other hand, higher thermogenesis could increase the risk of heat stress in early fed broilers. At d 2 after hatch, the delayed fed broilers preferred an ambient temperature of 29.4°C, which is in line with commercial recommendations of whole-house brooding temperature (Aviagen, 2018). At the same age, early fed broilers preferred an ambient brooding temperature of 27.2°C. In current recommended whole house brooding temperature setpoints no distinction is made between early and delayed fed broilers. Incorrect ambient brooding temperatures can negatively impact growth performance and increase ascites related mortality (Deaton et al., 1996; Lourens et al., 2005; Leksrisompong et al., 2009; Van der Pol et al., 2013). Strikingly, the difference in preferred ambient temperature between early and delayed fed broilers was still present at d 12, which suggests that feeding strategy post hatch may have long-lasting programming effects.

Incubation temperature x Feeding strategy

It was expected that early feeding would result in a lower temperature preference compared to delayed feeding when incubated at a control EST of 37.8°C, whereas this may not be the case when EST is lowered during late incubation, because a lower EST of 36.7°C during late incubation resulted in a higher preferred ambient temperature at d 1 (Wijnen et al., 2020). In the current study, no effect of lower EST on temperature preference was found and consequently the hypothesized interaction with feeding strategy was not found either.

De Jong et al. (2017) suggested that effects of early feeding on neonatal chick development may be dependent on incubation temperature. A lower EST of 36.7°C during late incubation resulted in a higher YFBM at hatch (Maatjens et al., 2016). We hypothesized that the need for early feed intake would be higher in chicks with a higher YFBM, because at hatch these chicks have less RY available relative to YFBM. This may explain why no interaction between EST and feeding strategy was found for any of the neonatal broiler development parameters.

Regardless of treatment group, 1st wk mortality was high with an average of 7.7%, This was probably the result of a bacterial infection, because necropsy showed that most broilers (approx. 70%) had ecolisepticemia and/or yolk sac infection. The old parental flock (54 wk) and lack of disinfection of hatching eggs, drinking water, and hatcher, may have increased the bacterial load (Yassin et al., 2009; Fertner et al., 2011; Poulsen et al., 2017). EST during late incubation tended to interact with feeding strategy on 1st wk mortality. First wk mortality tended (P=0.07) to be higher in the lower EST with early feeding group (13.7%) compared to the other 3 treatment groups (average 6.1%). The biological mechanism explaining this tendency is unclear. Possibly, early fed broilers were exposed to a relatively larger bacterial load and at an earlier age, because bacteria could replicate and spread via feces and drinking water. This risk seems to be no problem in good quality chicks, but in chicks with lower quality navels, as was currently observed in lower EST incubated female chicks, this might lead to higher mortality. A badly closed navel increases the risk of yolk sac infection and 1st wk mortality (Brandly, 1932; Dardiri et al., 1955; Fasenko and O'Dea, 2008; Olsen et al., 2012). This may also explain the higher mortality rate that was found after early feeding in another study (Lamot et al., 2016), whereas early feeding generally lowers mortality rates (de Jong et al., 2017). Yet, it should be emphasized that these are speculations based on a tendency for 1st wk mortality. Moreover, 1st week mortality was similar for both sexes, whilst a higher mortality for females would be expected as only females had worse navel conditions. Future studies on a potential relationship between incubation temperature and post hatch feeding strategy are required.

Incubation temperature

The finding that lower EST incubated chicks had higher corticosterone level at hatch compared to control EST indicated that lower EST may have caused cold stress to the embryo. Late term embryos are capable to show stress responses, because the hypothalamic-pituitary-

adenal axis becomes functional around d 14 of incubation (Case, 1952; McIlhone, 2011) and corticosterone level at hatch can be altered by thermal manipulations during incubation (Moraes et al., 2002; Amjadian and Shahir, 2020). Although avian embryos act poikilotherm during the majority of incubation time and full thermoregulatory response develops mainly after hatching (Dietz and van Kampen, 1994; French et al., 1997), embryos do decrease blood flow to the chorioallantois and increase heat production when eggs are cooled by 1°C or more (Romijn and Lonkhorst, 1955; Tazawa et al., 1988; Tazawa et al., 1989; Nichelman and Tzschentke, 1999; Lourens et al., 2006; Szdzuy et al., 2008). However, in the current study, these abilities appears to be insufficient, resulting in signs of cold stress when lowering EST during late incubation.

In conclusion, a lower EST during late incubation as well as delayed feed access after hatch appear to be stressful perinatal conditions. Also, early post hatch feeding strategy can have a long-lasting programming effect on thermoregulation and consequently on preferred ambient temperature during rearing.

ACKNOWLEDGEMENTS

The authors wish to thank Lagerwey hatchery for providing the eggs and HatchTech for funding this study. We would also like to thank the team members from Poulpharm and WUR colleagues that assisted during the experiment, with special attention for Ger de Vries Reilingh for her work on NAb, and Kevin Lin and Rudie Koopmanschap for their work on corticosterone. We are also grateful for the support of Thijme Hijink (HatchTech), Griet Vercauteren (Zoolyx), and Charlotte Visser and Bart de Bont (Wageningen University students).

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SUPPLEMENTARY FIGURE

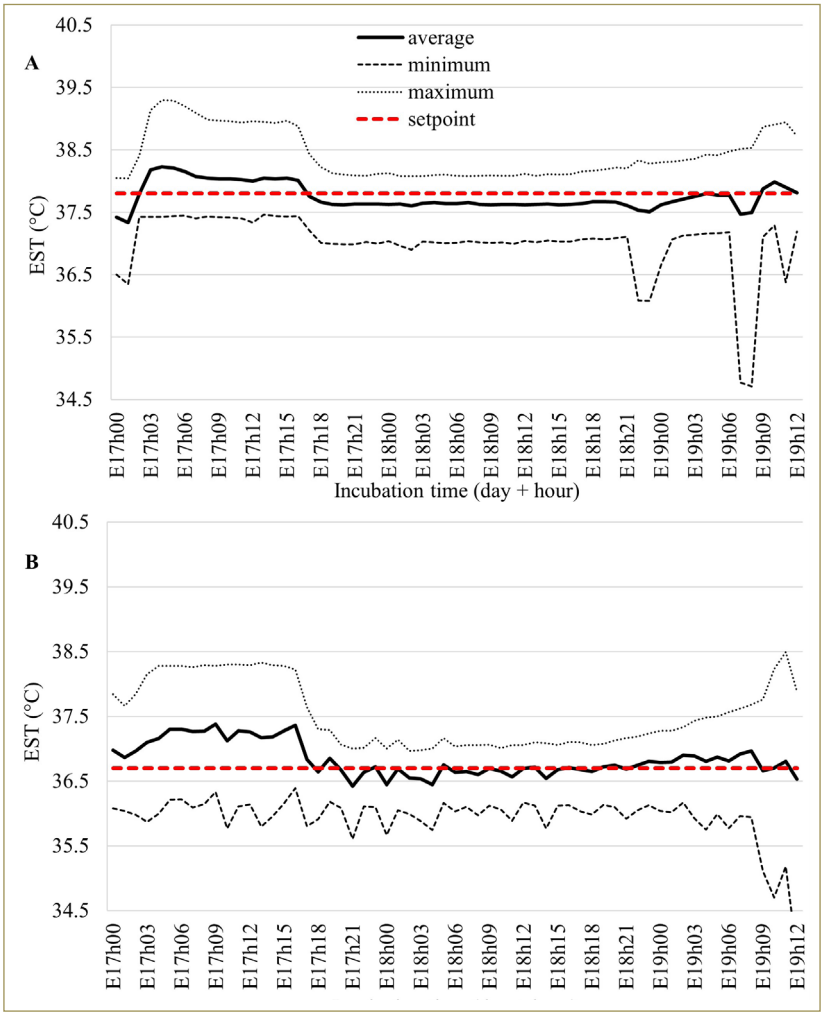

Figure S1. Actual average, minimum, and maximum eggshell temperature (**EST**) during embryonic d (**E**) 17 until E19 12h of broiler eggs incubated either A) at a setpoint of 37.8°C EST or B) at a setpoint of 36.7°C EST.

CHAPTER 5

LOW INCUBATION TEMPERATURE DURING LATE INCUBATION AND EARLY FEEDING AFFECT BROILER RESILIENCE TO NECROTIC ENTERITIS IN LATER LIFE

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Frontiers in Veterinary Science (2021) 8, 784869

ABSTRACT

Resilient animals can cope with environmental disturbances in life with minimal loss of function. Resilience can be enhanced by optimizing early life conditions. In poultry, eggshell temperature (**EST**) during incubation and early feeding are two early life conditions that are found to alter neonatal chick quality as well as immune response in later life. However, it has never been studied yet these early life conditions affect disease resilience of chickens at later ages. Hence, we studied effects of EST (37.8°C (**control**) or 36.7°C (**lower**)) during late incubation (≥embryonic day 17- 19.5) and feeding strategy after hatch (immediately (**early feeding**) or 51-54 h delayed (**delayed feeding**)) on later life broiler resilience in a 2 x 2 factorial arrangement. At hatch, 960 broilers of both sexes from a 54 wk old Ross breeder flock were equally divided over 32 pens (8 replicate pens / treatment combination) and grown for 6 wks. Necrotic enteritis was induced by a single inoculation of *Eimeria spp.* at d 21 and repeated *Clostridium perfringens* inoculation (3x /d) during d 21 - 25. Mortality and BW gain were measured daily during d 21 – 35 as indicators of resilience. Additionally, disease morbidity was assessed (gut lesions, dysbacteriosis, shedding of oocysts, footpad dermatitis, and natural antibody levels in blood). Results showed a lack of interaction between EST and feeding strategy for the vast majority of the variables. A lower EST resulted in lower BW gain at d 5 and 8 post *Eimeria* inoculation (P=0.02) and more *E. Maxima* oocysts in feces at d 8 post *Eimeria* inoculation compared to control EST (P<0.01). Early feeding tended to lower mortality compared to delayed feeding (P=0.06) but BW gain was not affected by feeding strategy. Morbidity characteristics were hardly affected by EST or feeding strategy. In conclusions, a few indications were found that a lower EST during late incubation as well as delayed feeding after hatch each may impair later life resilience to necrotic enteritis. However, these findings were not manifested consistently in all parameters that were measured and conclusions are drawn with some restraint.

INTRODUCTION

There is an increased global concern about the high use of antimicrobials and potentially related resistance threats (WHO, 2014). Efforts are made in various areas to reduce the usage of antibiotics. In animal husbandry, this reduction in antibiotics usage led to a rising interest in alternative approaches to enhance animal health, e.g. by enhancing animal resilience. Resilience can be defined as the capacity of an animal to absorb environmental disturbances and reorganize with minimal loss of function (Folke et al., 2010). In animal husbandry, environmental disturbances can be for instance changes in social structures, thermal conditions, or disease outbreaks. When focusing on the latter one, a resilient animal has a lower chance to become ill and once it does become ill it will show rapid recovery. Consequently, increased animal resilience may lead to a lower need of antibiotics, improved animal welfare, and beneficial revenues and sustainability. Resilience of animals is likely affected by early life conditions, because critical windows for immune organ development exists during early life (Renz et al., 2012) and environmental conditions in this period can affect the animal's immune system in later life (Luo et al., 2020).

In poultry, one environmental condition during early life that may affect chicken resilience is the embryo temperature during incubation. Embryos act poikilotherm and therefore their body temperature and, as a result, their metabolic rate and development is affected by their temperature. Embryo temperature is accurately reflected by eggshell temperature (**EST**) (French, 1997). Changes in EST patterns during incubation can affect chick quality at hatch and can even affect growth performance throughout life (Hulet et al., 2007; Tzschentke and Halle, 2009; Molenaar et al., 2011; Sozcu and Ipek, 2015; Lin et al., 2017). Moreover, EST can affect immunocompetence of broilers in later life by alterations peripheral lymphocyte numbers (Oznurlu et al., 2010), mucin expression and Salmonella Enteritidis colonization in the gut (de Barros Moreira Filho et al., 2015), and Newcastle disease vaccination response (Wijnen et al., 2020a). So far, a constant EST of 37.8°C throughout incubation is regarded optimal in terms of chick quality at hatch (Lourens et al., 2005). However, there are some indications that lowering EST to 36.7°C during late incubation may benefit embryo development in terms of yolk free body mass (Maatjens et al., 2016) and heart weight (Maatjens et al., 2016; Wijnen et al., 2020b). |However, other aspects of embryo development, such as chick length or bursa and intestine development, may be impaired by a lower EST during late incubation (Wijnen et al., 2020a - 2020b). Unfortunately, none of these studies investigated post-hatch broiler disease resistance and therefore possible effects of a lower EST during late incubation on broiler resilience against diseases in later life are unknown.

Another early life condition that may affect broiler resilience in later life is the provision of feed and water immediately after hatch moment (referred to as 'early feeding'). Currently, chicks are often withheld from feed and water during the period from hatch moment at the hatchery until placement at a farm. The duration of this period varies between batches of

chicks, but likely approximately 48 h is common whereas it was described to last up to 72 h in case of long transport duration (van de Ven et al., 2009; Willemsen et al., 2010a). Despite the availability of abdominal residual yolk during this period, withholding neonatal chicks from feed and water have been shown to result in BW loss and an impaired or delayed onset of gastrointestinal development (Noy and Sklan, 1999; Biloni et al., 2013; Lamot et al., 2014; Wang et al., 2020), thermoregulation (van den Brand et al., 2010), and immunocompetence (Shira et al., 2005; Simon et al., 2014; Price et al., 2015). Feeding chicks in this period will thus affect neonatal chick development, but is also known to have lasting effects on performance, mortality, and immune response after the period of feed deprivation (Shira and Sklan, 2005; de Jong et al., 2017; Juul-Madsen et al., 2004; Lamot et al., 2016). Most studies related to early feeding provided vaccines or model antigens to induce a disease response rather than inducing a disease. Therefore, based on these studies, no definite conclusion can be drawn on whether possible alterations in immune response found by model antigens result in altered broiler resilience. The use of disease models in studies could elucidate this, especially if kinetics in functional losses or pathogenesis are determined. Until now, such studies are very limited (Ao et al., 2012; Simon et al., 2015).

This study aimed to investigate effects of EST during late incubation and feeding strategy immediately post hatch on later life resilience. Both factors may interact with each other as EST may for instance affect intestinal morphology and digestive enzyme activity in such a way that a chick is better prepared for exogenous feed intake at hatch moment and will have a higher benefit from early feeding.

MATERIAL AND METHODS

This study was conducted during April to May 2019 at Wageningen University & Research, The Netherlands (incubation and hatch period) and Poulpharm, Zwevegem, Belgium (grow out period). The experimental protocol from hatch until transport of day-old chicks was approved by the Governmental Commission on Animal Experiment, The Hague, The Netherlands, approval number: 2018.W-0020.001. The experimental protocol from transport until the end of the experiment were carried out according to the recommendations and following approval of the Ethical Committee of Poulpharm, Belgium, approval number P19034-FP.

Experimental design

The experiment was set up as a 2 x 2 factorial arrangement with EST during late incubation and feeding strategy after hatch as treatments. EST from embryonic day (**E**) 17 until E19.5 was set at 37.8°C (**control**) or at 36.7°C (**lower**), whereas feeding strategy included

access to feed and water within 3 to 6 h after hatch (**early feeding**) or within 51 to 54 h after hatch (**delayed feeding**).

Egg selection

Eggs from a 54-wk-old Ross 308 broiler breeder flock were stored at a commercial hatchery (Lagerwey BV, Lunteren, The Netherlands) for 4 days at 20°C. All eggs were laid on the same date in 2 broiler houses at one farm. Both houses were held under similar environmental and management conditions and eggs coming from both houses were randomly mixed between treatments. To exclude potential effects of initial egg weight (Wilson, 1991), 10 egg trays with 150 eggs each were bulk-weighed to determine the average egg weight (70.1 g). Three equal weight classes within 1.5 g of the average egg weight were determined (69.35-69.85, 69.86-70.35, 70.36-70.85). Thereafter, eggs were weighed individually until 446 first-grade eggs (clean, without hairline cracks or malformations) per weight class were selected (total 1,338 eggs). Eggs were transported for approximately 30 min to the animal research facility of Wageningen University & Research (Wageningen, The Netherlands), where eggs of each weight class were equally divided over 16 setter trays (type 88 Setter Tray, HatchTech, Veenendaal, The Netherlands).

Incubation

All 16 trays were set in one incubator (type climate respiration chamber, details provided by (Heetkamp et al., 2015). Four EST sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK) were attached to the equator of the eggshell of 4 individual eggs, using silicone heat sink compound (Type 340; Dow Corning, Midland, MI) and a small piece (approx. 1.5 x 1.5 cm) of elastic performance tape (Leukotape K, Essity, Hamburg, Germany). A 22-h preincubation warming profile was applied before the onset of incubation, adapted from (van Roovert-Reijrink et al., 2018) such that eggs were linearly warmed from storage temperature to 30.6°C EST in 5 h and from 30.6°C to 37.8°C EST in 17 h. The moment eggs reached an EST of 37.8°C was considered to be the start of incubation (E0). Until E17, incubator temperature was continuously adjusted, based on the median temperature of the 4 EST sensors to aim at an EST of 37.8°C. Eggs were turned every hour by an angle of 45° from horizontal. Relative humidity was maintained between 50 and 55%, and CO_2 level was maintained below 3,500 ppm.

At E17, all eggs were candled and eggs containing a viable embryo (N=1,072; 86.7% of fertile eggs at set) were transferred to hatching baskets. Eggs were transferred to one hatcher basket per setter tray. These hatcher baskets were divided over 4 incubators (4 baskets / incubator). Two incubators were aimed at an EST of 37.8°C (control EST), whereas the other 2 incubators aimed at an EST of 36.7°C (lower EST). EST control in all four incubators was performed as described above. Relative humidity was maintained between 45 and 75% and CO_2 levels were maintained below 3,500 ppm. After 468 h of incubation (E19 12h), the

incubator temperatures were fixed at their actual setting, and EST was allowed to change as chicks started to emerge from the eggshell. Details about actual EST are provided in (Wijnen et al., 2022).

Hatch and early feeding

From 468 h of incubation onward, every 3 h the incubators were opened to check whether chicks had hatched. A chick that was fully emerged from the shell was marked with a colored dot on the head using a permanent marker. After marking, chicks were placed back in their original hatcher basket to dry. Three h after a chick was marked, it was pulled from the incubator and classified either as 2nd grade chick if any abnormality was observed (e.g. crossed beak, blindness, exposed brains, 4 legs, exposed yolk) or as 1st grade chick (all remaining chicks). All 2nd grade chicks were excluded from the experiment. The 1st grade chicks (N=1,028) were feather sexed, marked with a plasticized paper neck tag (size 5 x 2 cm), and transferred to HatchCare hatcher baskets (HatchTech, Veenendaal, The Netherlands). In half of these baskets (early feeding treatment), ad libitum starter pellet feed (details given in 'grow out' section below) and fresh water were provided. The other half of these baskets had empty gutters and troughs (delayed feeding treatment). Chicks were stored in these hatcher baskets in a chick storage room at 36.0°C and 55% RH without forced airflow until E21 12 h. At E21 12 h, all water gutters were emptied and baskets (incl. residual feed in baskets from early feeding treatment) were transferred to a climate-controlled van designed for chicken transportation at 29.4°C and 36% RH with forced airflow (Chickliner, Renswoude, The Netherlands). Chicks were transported during approximately 3 h to a grow out facility from Poulpharm, Zwevegem, Belgium.

Grow out

Layout

At arrival at the growout facility (regarded as day 0), 960 broiler chicks were selected randomly and divided over 32 floor pens in one broiler house (8 replicate pens / treatment). Pens were divided over 8 equal blocks (4 pens / block) and all four treatments were allocated to each block. At placement, each pen contained 15 male and 15 female broilers. Pen size was 260 x 105 cm and fenced with 60 cm high solid mesh. The concrete pen floor was covered with a 1 cm think layer of wood shavings. Each pen contained 4 drinking nipples and one metal feed silo (35 cm diameter).

Delayed feeding

At placement, pens from the delayed feeding treatment were temporarily divided with a fence in the middle, which divided the pen into an unfed side and a fed side. The unfed side floor was covered with cardboard to prevent litter consumption. At placement, all broilers within these pens were positioned in the unfed side of the pen. Thereafter, each broiler was

relocated individually to the fed side of the pen 48 h after it had received its neck tag. As a result, delayed fed broilers had access to feed and water within 51 to 54 h post hatch. Fences and cardboard were removed after all broilers were relocated to the fed side of the pen. Which treatment was contained in a pen was blinded from that moment onward.

1.5.3 Feed and vaccinations

A starter pelleted diet (ME broiler = 2,838 kcal/kg, CP = 221.8 g/kg, digestible lysine = 12.9 g/kg) with a diameter of 2.6 mm was provided from d 0 until d 13, a grower pelleted diet (ME broiler = 2,999 kcal/kg, CP = 269.4 g/kg, digestible Lysine = 15.17 g/kg) with a diameter of 3.2 mm from d 13 until d 28, and a finisher pelleted diet (ME broiler = 3,001 kcal/kg, CP = 188.6 g/ kg, digestible Lysine = 10.85 g/kg) with a diameter of 3.2 mm was provided from d 28 onward. Diets did not contain coccidiostats and were produced by Research Diet Services (RDS, Wijk bij Duurstede, The Netherlands) according to the guidelines of the Federation Dutch Animal Feed chain (CVB, 2016). Feed and water were provided ad libitum. At d 0, broilers were spray vaccinated against Newcastle disease (Avishield ND) and infectious bronchitis (Poulvac IB primer). At d 12, Newcastle disease vaccination (Avishield ND) and Gumboro disease vaccination (Nobilis Gumboro D78) were provided via drinking water.

1.5.4 Climate

Ambient temperature setpoint was 35°C at placement and was linearly decreased to 20°C at d 24 and this setpoint was maintained until the end of the study. As a difference in ambient temperature preference was expected between early and delayed fed broilers, a heat lamp was provided in the middle of each pen from placement until d 12, meaning that each broiler could choose its own preferred ambient temperature. Relative humidity was on average 34.5% and varied between 20 and 50%. At placement, 24 h of light was provided and from d 2 onward, 1 h of darkness / 24 h was added each day until 6 h of darkness / 24 h was provided by d 7 and this lighting schedule was maintained until the end of the study.

Necrotic enteritis model

A subclinical necrotic enteritis (**NE**) was induced by adapting a protocol from (Van Waeyenberghe et al., 2016). Fish meal (10%) and rye (5%) were included into the grower diet (d 13 - 28) as predisposing factors for NE induction. At d 21, all broilers were orally inoculated with 1 ml inoculum containing field isolates of *Eimeria Acervulina* (80,000 oocysts), *Eimeria Maxima* (40,000 oocysts), and *Eimeria Mitis* (5,800 oocysts). These field isolates were purified and differentiated out of samples from Germany, Australia, and Germany, respectively. At the day of *Eimeria* inoculation and 4 days thereafter (d 21 – 25), all broilers were also orally inoculated 3x / d (approximately 8:00 am, 11:00 am, and 02:00 pm) with approximately $1x10^9$ colony-forming units of a *netB*-positive *Clostridium Perfringens* strain 56 (Gholamiandekhordi et al., 2006; Timbermont et al., 2009). *Clostridium Perfringens* strain was streaked onto

Columbia Sheep-blood agar (37°C, anaerobic incubation for approximately 18h). Following, different isolated colonies were selected for further constitution of the bacterial inoculum. Colonies were aseptically transferred into Brain-Heart Infusion (BHI) medium and incubated overnight (37°C, anaerobic incubation for approximately 15h). The number of CFU/mL in the final inoculum composition was calculated based on viable cell counts, after streak plating a 10-fold dilution series of the inoculum onto Columbia Sheep-blood agar. All plates were incubated anaerobically at 37°C for approximately 18-24h.

Data collection

The response of broilers to the NE induction was monitored from the day of *Eimeria* inoculation (d 21) until the end of the experiment (d 35) by determining mortality and BW gain as indicators of resilience. Disease morbidity was assessed by determining NE lesions, coccidiosis, dysbacteriosis, oocyst shedding, natural antibody levels in blood, and footpad dermatitis.

Mortality was determined by daily monitoring of dead broilers each morning from the day of *Eimeria* inoculation until the end of the experiment. Broilers were observed daily to check their health and wellbeing and were culled by caretakers if a humane endpoint was reached, as defined by (Marchewka et al., 2013).

Changes in BW were measured to determine functional loss. From d 21 up to and including d 31 and once finally at d 35, all broilers were weighed individually during the morning. Average daily gain (**ADG**) was calculated for each broiler that survived during the experiment.

Necrotic lesions, coccidiosis, and dysbacteriosis were scored by dissecting broilers approx. 3 h before *Eimeria* inoculation and at d 6, 7, and 14 post *Eimeria* inoculation (**PEI;** respectively d 21, 27, 28, and 35 post hatch). All scores were performed blind by trained veterinarians. At approx. 3 h before *Eimeria* inoculation, a varying number of broilers was dissected per pen such that the remaining number of broilers in each pen was 20 of equal sex ratio. Thereby possible effects of varying animal densities between pens, caused by mortality between hatch and onset of NE, on outcome of NE response was prevented. This procedure resulted in dissection of in total 100 broilers at approx. 3 h before *Eimeria* inoculation. To ensure that the remaining living broilers (20 / pen) were closest to treatments average, broilers to be used for dissection (n= 100) were selected as follows. The average BW at d 14 post hatch was calculated per sex per treatment group. Subsequently, within each pen and per sex, the first broiler that was selected for dissection had the most deviating higher weight at d 14 than treatment group average. The second broiler that was selected had the most deviating lower weight than treatment group average and so on until 20 broilers per pen remained. At d 6 and 7 PEI, 10 broilers per pen (equal sex ratio) were dissected (5 broilers / pen / d). This time broilers within each pen were selected for dissection if their BW at d 25 deviated the most from the average BW of similar sex and treatment group. At d 14 PEI, 5 broilers per pen

were dissected (3 females and 2 males). Broilers were selected based on their BW at d 31 as described for d 6 and 7 PEI.

Necrotic lesions were scored according to protocol (Keyburn et al., 2006). At d 6 and 7 PEI, all dissected broilers were scored on macroscopic NE lesions in the small intestine (duodenum to ileum) on a severity scale from 0 (= no gross lesions) to 6 (= diffuse necrosis). Broilers with NE lesion scores of 2 or more were considered as NE positive and incidence of NE was based on this classification.

Coccidiosis was determined in all dissected broilers during all dissection moments according to protocol (Johnson and Reid, 1970). *E. Acervulina*, *E. Maxima*, and *E. Tenella* macroscopic lesions were each scored on a severity scale 0 (= no gross lesions) to 4 (= severe lesions). The total mean lesion score (**TMLS**) was calculated by summing up scores of each *Eimeria* type, resulting in a TMLS severity score between 0 and 12 (De Gussem, 2007). Incidence was calculated by classifying broilers with TMLS of 1 or more as coccidiosis positive.

Dysbacteriosis was scored according to protocol (Teirlynck et al., 2011). At the day of *Eimeria* inoculation and d 14 PEI, in all dissected broilers the absence (= score 0) or presence (= score 1) of gut ballooning, undigested feed particles, redness, gut wall thickness, flaccidity, and abnormal lumen content was determined. Dysbacteriosis severity score was calculated by summing up all scores, resulting in a dysbacteriosis severity score between 0 and 10. Incidence was calculated by classifying broilers with dysbacteriosis score of 3 or more as dysbacteriosis positive.

Oocyst shedding was assessed in all pens at one day before *Eimeria* inoculation and at d 4 and 7 PEI (respectively d 20, 25, and 28 post hatch). Several fresh droppings (approximately 200 g) were collected and mixed per pen. Oocysts per gram faeces (**OPG**) from *E. Acervulina*, *E. Maxima*, *E. Mitis*, *E. Tenella*, *E. Brunetti*, and *E. Necatrix/praecox* were counted according to the McMaster method (Gordon and Whitlock., 1939). The *Eimeria spp*. differentiation was based on morphometrical examination of each oocyst according to protocol (Reid and Long, 1979) performed by trained and validated parasitology lab technicians. *E. Brunetti* and *E. Necatrix/praecox* were not detected at any collection day and are therefore not discussed any further in this paper. One day before *Eimeria* inoculation, *E. Acervulina*, *E. mitis*, and *E. Tenella* oocysts were found in 75.0, 62.5, and 6.3% of the pens, respectively. Preliminary analysis showed that there was no effect of treatment on OPG prior to *Eimeria* inoculation nor at d 5 or 8 PEI for any of these three *Eimeria* species. The OPG of these *Eimeria* species were excluded from the results as their presence 1 d before inoculation indicated that OPG numbers at d 5 and 8 PEI could not be attributed solely to the disease challenge.

At d 0, 6, and 14 PEI (respectively d 21, 27, 35 post hatch), blood was collected from the wing vein of 2 broilers per pen (1 male and 1 female) randomly chosen from the ones that were selected for dissection. Blood was collected in natrium heparinized tubes (Vacuette 4 mL FX, Greiner Bio-One), stored on ice, and plasma was collected after centrifugation at

2,000 x g for 10 min. Plasma was stored at -20°C until samples were analyzed for the level of IgY and IgM natural antibodies (**NAb**) though the amount of immunoglobulin binding to keyhole limpet hemocyanin (**KLH**) adjusted from the protocol of (Lammers et al., 2004). Briefly, 96-well medium binding flat-bottomed plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were coated with 100 µL coating buffer (5.3 g/L Na_2CO_3, and 4.2 g/L $NaHCO_3$; pH 9.6), containing either 2 or 4 µg/mL KLH (H8283, Sigma-Aldrich, St. Louis, MO, USA) for isotypes IgY and IgM respectively. Plates were incubated at 4°C overnight, washed with tap water containing 0.05% Tween 20, and tapped dry. Plasma samples were 1:10 pre-diluted (both isotypes) with dilution buffer (phosphate buffered saline [PBS; 10.26 g/L $Na_2HPO_4 \cdot H_2O$, 2.36 g/L KH_2PO_4, and 4.50 g/L NaCl; pH 7.2], containing 1% horse serum, and 0.05% Tween 20). Pre-dilutions were further diluted with dilution buffer such that tested plasma dilution were 1:40, 1:160, 1:640, and 1:2,560 for both isotypes. Plates were incubated for 1.5 h at room temperature and then washed again with tap water, containing 0.05% Tween 20. Subsequently, plates were incubated for 1.5 h at room temperature with 1:20,000-dilutions of either goat-anti-chicken IgG(Fc) (Bethyl A30-104P) or goat anti-chicken IgM (Bethyl A30-102P), each labeled with horse radish peroxidase (polyclonal antibodies from Bethyl Laboratories, Montgomery, TX, USA). Plates were washed again with tap water, containing 0.05% Tween 20 after which antibodies to KLH were visualized by adding 100 µL substrate buffer (tetramethylbenzidine + 0.05% H_2O_2). After 20 min, the reaction was stopped with 50 µL of 1.25 M H_2SO_4 and extinctions were measured at 450 nm with a microplate spectrophotometer (Multiskan Go, Thermo scientific, Breda, The Netherlands). Antibody titers were calculated based on log2 values of the dilutions that gave extinction closest to 50% of E_{MAX}, where E_{MAX} represents the mean of the highest extinction of the standard positive plasma samples, thereby partly correcting for plate to plate differences (van der Klein et al., 2015).

Footpad dermatitis (**FPD**) for both feet was assessed in all dissected broilers at all days, using the protocol of (Ekstrand et al., 1998). Macroscopic lesions were scored blind by trained veterinarians either 0 (=no lesions, only mild hyperkeratosis, no discoloration or scars), 1 (= mild lesions; superficial lesions, erosions, papillae and discoloration of the footpad), or 2 (= severe lesions; deep lesions, ulcers, and scabs). The highest score from both feet was noted as final score. A score 2 was only found once (d 35). Therefore, score 1 and 2 were merged and scored 1 and classified as FPD positive.

Statistical analyses

All data were analyzed, using the statistical software package SAS (Version 9.4, SAS institute, 2010). The basic model used for all data was

$$Y_{ij} = \mu + EST_i + FEED_j + EST \times FEED_{ij} + e_{ij}, \quad [1]$$

where, Y_{ij} = the dependent variable, μ = the overall mean, EST_i = eggshell temperature during late incubation ($_i$ = 36.7°C or 37.8°C), $FEED_j$ = feeding strategy ($_j$ = early or delayed), EST x $FEED_{ij}$ = the interaction between EST during late incubation and feeding strategy, and e_{ij} = the error term.

Pen was considered as the experimental unit for OPG and mortality data. Preliminary analysis showed that survival lines in a Cox proportional hazard model (PhReg) crossed and thus assumptions of overall homogeneity of survival distributions were not met. Alternatively, percentage of broilers that died or culling percentage were both calculated per pen by summing up the number of broilers that died or were culled PEI divided by the number of broilers before *Eimeria* inoculation (20/pen) minus the number of broilers dissected for morbidity scores at d 6 and 7 PEI (10/pen). Total mortality was calculated as the sum of broilers that died PEI and were culled PEI.

All remaining data was collected for individual broilers, but pen was still considered as the experimental unit by extending model 1 with pen (1-32) as a random factor. Sex was added to model 1 as fixed factor. Besides, sex and treatment interactions were added (sex x EST, sex x FEED, sex x EST x FEED). Preliminary statistical analysis did not show significant effects of sex x EST or sex x EST x FEED for any of the variables and therefore these interactions were deleted from the model. A significant interaction between sex x FEED was only found for FPD at d 6 PEI and sex x FEED was therefore added to model 1 for this single dependent variable only. In all other cases, interactions between sex and FEED were also excluded from the model.

The PROC MIXED procedure was used to analyze mortality data (died, culled, total mortality), changes in BW (BW, ADG), OPG, and natural antibodies. Model assumptions were verified by inspection of residual plots and not normally distributed data were log transformed. For BW during d 0 to 10 PEI, broiler was the repeated subject and model 1 was extended with day and the interactions between day and EST, day and FEED, and day and sex. Covariance structure was selected based on best model fit according to smallest Akaike's information criteria, resulting in a toeplitz covariance structure. Data is expressed as LSmeans ± SEM. Tukey adjustments for multiple comparisons were used to compare least square means. A P-value ≤0.05 was considered as significant and a P-value >0.05 and ≤ 0.10 as a tendency.

The PROC GLIMMIX procedure was used to analyze severity and incidence of NE lesions, coccidiosis, dysbacteriosis, and FPD. Severity scores were analyzed with a multinomial cumulative logit link function in model 1. Incidence was analyzed with a binary logit link function in model 1. For NE incidence, day (6, 7, 14 PEI) was added as a fixed factor to model 1 and the veterinarian that performed the observation as a random factor. For FPD, bodyweight was added as a covariate. Data is expressed as mean ± SE. A P-value ≤0.05 was considered as significant and a P-value >0.05 and ≤ 0.10 as a tendency.

RESULTS

Mortality

Total mortality PEI (dead + culled) was on average 17.5% (N= 56), from which 15.3% occurred within 1 wk PEI. The percentage of dead or culled broilers PEI did not show an interaction between EST and feeding strategy (Table 1; $P \geq 0.28$), nor a main effect of EST ($P \geq 0.48$) or feeding strategy ($P \geq 0.11$). Total mortality PEI also did not show an interaction between EST and feeding strategy ($P=0.46$), nor showed a main effect of EST ($P=0.68$). However, early feeding tended to result in a lower total mortality PEI compared to delayed feeding ($\Delta=6.6\%$; $P=0.06$).

Table 1. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic day 17 – 19.5; 37.8°C (**control**) or 36.7°C (**lower**)) and/or feeding strategy after hatch (immediate access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on mortality of broilers during 2 wks following necrotic enteritis induced at d 21 post hatch (LSmeans ± SEM).

	N[1]	Died (%)	Culled (%)	Total mortality[2] (%)
EST				
Control	16	9.9	4.9	14.8
Lower	16	15.0	3.1	18.1
SEM		2.75	1.76	3.12
Feeding strategy				
Early	16	10.6	2.6	13.1
Delayed	16	14.3	5.4	19.7
SEM		2.75	1.76	3.12
EST x Feeding strategy				
Control x Early	8	7.9	2.5	10.4
Control x Delayed	8	11.9	7.3	19.2
Lower x Early	8	13.3	2.6	15.9
Lower x Delayed	8	16.7	3.5	20.2
SEM		3.89	2.49	4.38
P-values				
EST		0.56	0.48	0.68
Feeding strategy		0.11	0.39	0.06
EST x Feeding strategy		0.28	0.47	0.46

[1] Number of pens.
[2] Total mortality = died + culled.

Body weight changes

Regardless of the day PEI, BW did not show an interaction between EST and feeding strategy (P=0.30), nor showed a main effect of EST (Figure 1A; P=0.37). Early feeding resulted in higher BW at all days compared to delayed feeding (Figure 1B; P=0.04). Males had higher BW compared to females at any d PEI (P<0.01; data not shown).

Regardless of the day PEI, ADG did not show an interaction between EST and feeding strategy (P=0.13), nor a main effect of feeding strategy (Figure 2B; P=0.49). Lower EST resulted in a lower ADG at d 5 and 8 PEI (Figure 2A; P=0.02 both days) and tended to result in a lower ADG at d 6 PEI (P=0.06) compared to control EST. Males had higher ADG compared to females at any day PEI (P<0.01; data not shown).

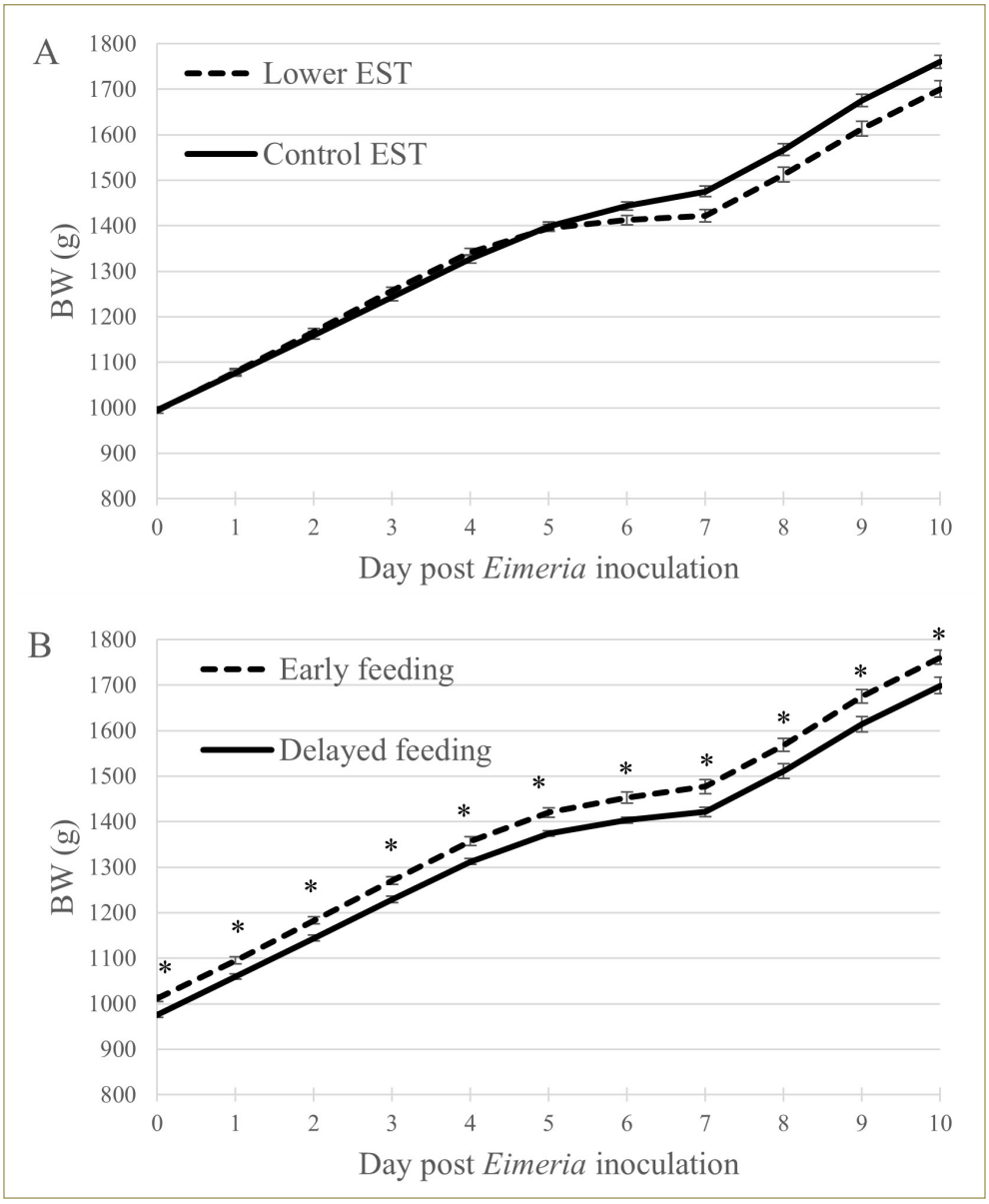

Figure 1. **A**) Effect of eggshell temperature (**EST**) during late incubation (≥embryonic day 17 – 19.5; 37.8°C (**control**) or 36.7°C (**lower**)) or **B**) effect of feeding strategy after hatch (immediate access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on broiler BW during 10 days post *Eimeria* inoculation (d 21 post hatch) to induce necrotic enteritis (N=16 pens / treatment). An asterisk (*) indicates significant difference ($P<0.05$) between LSmeans of treatments within day post *Eimeria* inoculation. Error bars indicate standard error.

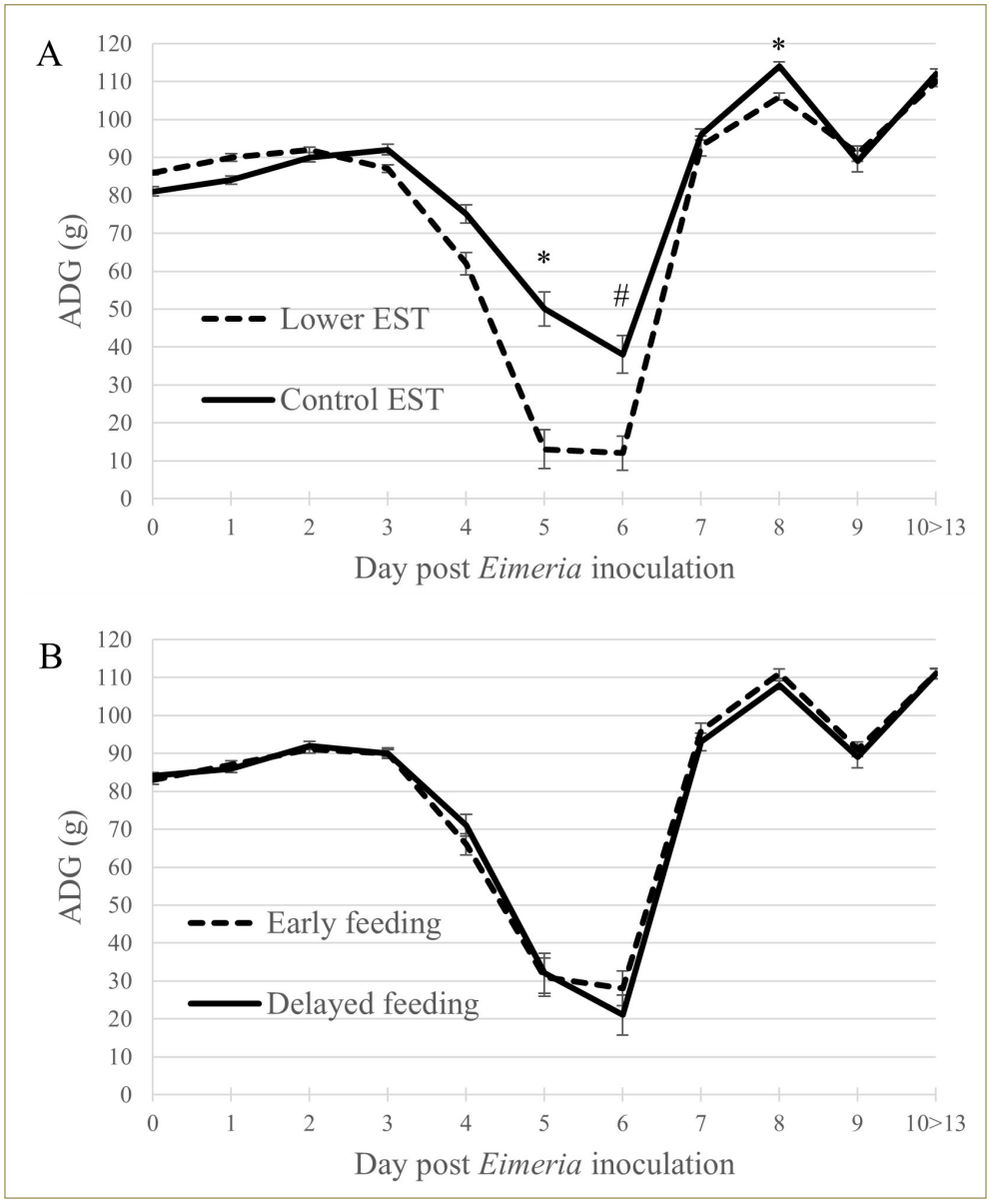

Figure 2. **A**) Effect of eggshell temperature (**EST**) during late incubation (≥embryonic day 17 – 19.5; 37.8°C (**control**) or 36.7°C (**lower**)) or **B**) effect of feeding strategy after hatch (immediate access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on broiler average daily gain during 13 days post *Eimeria* inoculation (d 21 post hatch) to induce necrotic enteritis (N=16 pens / treatment). An asterisk (*) indicates significant difference ($P<0.05$) between LSmeans of treatments within day post *Eimeria* inoculation and a hashtag (#) indicates a tendency to differ ($P<0.10$). Error bars indicate standard error.

Necrotic enteritis lesions

NE lesion severity was worse on d 6 PEI compared to d 7 PEI (Δ=1.0 score; $P<0.01$) respectively). Neither on d 6 PEI nor on d 7 PEI, severity of NE lesions showed an interaction between EST and feeding strategy (Table 2; $P\geq0.31$) nor a main effect of EST ($P\geq0.46$) or feeding strategy ($P\geq0.43$). NE incidence was on average 84.4%, which was similar on d 6 and 7 PEI ($P=0.27$) and did not show an interaction between EST and feeding strategy ($P=0.31$), nor show a main effect of EST ($P=0.51$) or feeding strategy ($P=0.14$). At d 7 PEI, males tended to have more severe NE lesions compared to females (Δ=0.4 score; $P=0.09$) , but at d 6 PEI severity of NE lesions was not different between sexes ($P=0.26$). NE incidence was not different between sexes ($P=0.83$).

Table 2. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic day 17 – 19.5; 37.8°C (**control**) or 36.7°C (**lower**)) and/or feeding strategy after hatch (immediate access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on necrotic lesions in the small intestine of broilers at d 6 or 7 post *Eimeria* inoculation (d 21 post hatch) to induce necrotic enteritis (mean ±SE).

	N[1]	NE severity (score 0-6) day post *Eimeria* inoculation[2]		NE incidence[3] (%)
		6	7	
EST				
Control	16	3.8 ±0.24	3.0 ±0.16	82.9 ±3.05
Lower	16	4.0 ±0.23	2.8 ±0.15	85.4 ±2.87
Feeding strategy				
Early	16	3.9 ±0.22	2.9 ±0.14	87.7 ±2.65
Delayed	16	3.9 ±0.25	2.8 ±0.17	80.5 ±3.24
EST x Feeding strategy				
Control x Early	8	3.6 ±0.34	2.9 ±0.20	84.2 ±4.18
Control x Delayed	8	4.0 ±0.33	3.0 ±0.25	81.6 ±4.45
Lower x Early	8	4.2 ±0.28	2.9 ±0.20	91.0 ±3.24
Lower x Delayed	8	3.8 ±0.37	2.6 ±0.23	79.5 ±4.73
P-values				
EST		0.91	0.46	0.51
Feeding strategy		0.87	0.43	0.14
EST x Feeding strategy		0.50	0.31	0.31
Sex		0.26	0.09	0.83

[1] Number of pens.
[2] 5 broilers / pen / day.
[3] Broilers with lesion scores of 2 or more were classified as NE positive.

Coccidiosis

Neither on the day of *Eimeria* inoculation, nor on d 6 PEI or d 7 PEI, severity (TMLS) or incidence (%) of coccidiosis showed an interaction between EST and feeding strategy (Table 3; $P \geq 0.34$), nor a main effect of EST ($P \geq 0.23$) or feeding strategy ($P \geq 0.58$). At d 14 PEI, lower EST resulted in a lower coccidiosis severity ($\Delta = 0.3$ TMLS; $P = 0.01$) and tended to result in a lower coccidiosis incidence ($\Delta = 15.7\%$; $P = 0.09$) compared to control EST. Severity and incidence of coccidiosis at d 14 PEI both did not show an interaction between EST and feeding strategy ($P \geq 0.30$), nor a main effect of feeding strategy ($P \geq 0.20$). There was no difference in severity or incidence of coccidiosis between sexes ($P \geq 0.27$).

Dysbacteriosis

Neither on the day of *Eimeria* inoculation nor on d 14 PEI, severity or incidence (%) of dysbacteriosis showed an interaction between EST and feeding strategy (Table 4; $P \geq 0.34$), nor a main effect of EST ($P \geq 0.40$) or feeding strategy ($P \geq 0.25$). There was no difference in severity or incidence of dysbacteriosis between sexes on either dissection days ($P \geq 0.63$).

Oocyst shedding

Neither one day before *Eimeria* inoculation nor at d 5 PEI , *E. Maxima* OPG was found in any of the pens. *E. Maxima* OPG at d 8 PEI did not show an interaction between EST and feeding strategy (Figure 3; $P = 0.79$), nor a main effect of feeding strategy ($P = 0.87$), but lower EST resulted in a higher *E. Maxima* OPG at d 8 PEI compared to control EST ($\Delta = 32{,}638 \pm 4117$ OPG; $P < 0.01$).

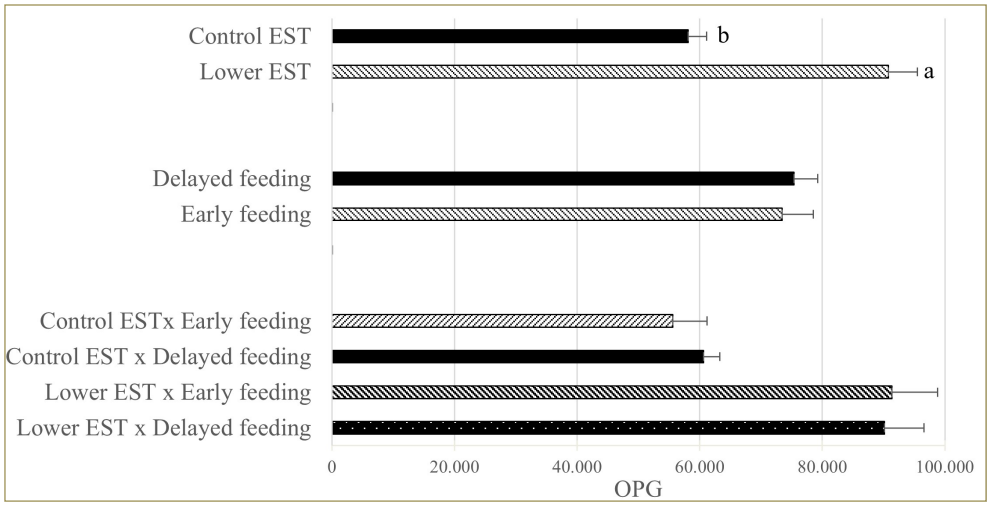

Figure 3. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic day 17 – 19.5; 37.8°C (**control**) or 36.7°C (**lower**)) and/or feeding strategy after hatch (immediate access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on *E. Maxima* oocyst per gram feces (**OPG**) at d 8 post *Eimeria* inoculation (d 21 post hatch) to induce necrotic enteritis (N=16 pens / treatment). a-b Least squares means within a factor lacking a common superscript differ (P<0.05). Error bars indicate standard error.

Table 3. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic day 17 – 19.5; 37.8°C (**control**) or 36.7°C (**lower**)) and/or feeding strategy after hatch (immediate access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on coccidiosis of broilers at d 0, 6, 7, or 14 post *Eimeria* inoculation (d 21 post hatch) to induce necrotic enteritis (mean ±SE).

	N[1]	Coccidiosis severity (TMLS[2] 0-12) day post *Eimeria* inoculation[4]				Coccidiosis incidence[3] (%) day post *Eimeria* inoculation[4]			
		0	6	7	14	0	6	7	14
EST									
Control	16	2.2 ±0.18	3.8 ±0.24	2.8 ±0.20	0.9 ±0.10[a]	85.3 ±4.17	92.1 ±3.09	92.1 ±3.09	70.0 ±5.12
Lower	16	1.9 ±0.21	4.1 ±0.23	3.2 ±0.21	0.6 ±0.07[b]	90.6 ±5.15	96.1 ±2.21	96.0 ±2.29	54.3 ±5.53
Feeding strategy									
Early	16	2.2 ±0.23	3.8 ±0.22	3.0 ±0.20	0.8 ±0.10	86.0 ±5.28	94.8 ±2.53	94.8 ±2.53	55.6 ±5.52
Delayed	16	2.0 ±0.17	4.0 ±0.25	3.0 ±0.21	0.9 ±0.08	87.7 ±4.13	93.4 ±2.84	93.2 ±2.96	68.8 ±5.18
EST x Feeding strategy									
Control x Early	8	2.4 ±0.26	3.7 ±0.33	2.8 ±0.30	1.0 ±0.16	86.1 ±5.76	94.7 ±3.62	92.1 ±4.37	67.5 ±7.41
Control x Delayed	8	2.1 ±0.24	3.8 ±0.35	2.9 ±0.26	1.0 ±0.12	84.4 ±6.02	89.5 ±4.98	92.1 ±4.37	72.5 ±7.06
Lower x Early	8	1.6 ±0.40	4.0 ±0.30	3.2 ±0.26	0.5 ±0.10	85.7 ±13.23	94.9 ±3.53	97.4 ±2.53	43.9 ±7.75
Lower x Delayed	8	2.0 ±0.24	4.1 ±0.36	3.1 ±0.32	0.8 ±0.10	92.0 ±5.43	97.4 ±2.60	94.3 ±3.92	65.0 ±7.54
P-values									
EST		0.23	0.68	0.34	0.01	0.79	0.46	0.35	0.09
Feeding strategy		0.87	0.79	0.99	0.38	0.58	0.92	0.59	0.20
EST x Feeding strategy		0.34	0.85	0.53	0.30	0.80	0.61	0.57	0.38
Sex		0.31	0.57	0.90	0.39	0.77	0.84	0.27	0.54

[a-b] Means within a column and factor lacking a common superscript differ (P<0.05).

[1] Number of pens.

[2] TMLS= Total mean lesion score. Sum of coccidiosis score for *E. Acervulina*, *E. Maxima*, *E. Tenella*.

[3] Broilers with TMLS of 1 or more were classified as coccidiosis positive.

[4] In total 100, 153, 150, and 161 broilers for d 0, 6, 7, and 14, respectively.

Table 4. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic day 17 – 19.5; 37.8°C (**control**) or 36.7°C (**lower**)) and/or feeding strategy after hatch (immediate access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on dysbacteriosis of broilers at d 0 or 14 post *Eimeria* inoculation (d 21 post hatch) to induce necrotic enteritis (mean ±SE).

		Dysbacteriosis severity (score 0-10)		Dysbacteriosis incidence[2] (%)	
		day post *Eimeria* inoculation[3]		day post *Eimeria* inoculation[3]	
	N[1]	0	14	0	14
	N[1]	6	14	6	14
EST					
Control	16	2.9 ±0.15	2.8 ±0.15	33.8 ±5.80	33.8 ±5.29
Lower	16	2.8 ±0.19	2.9 ±0.14	21.9 ±7.31	35.8 ±5.33
Feeding strategy					
Early	16	2.7 ±0.19	2.9 ±0.15	25.6 ±6.65	35.8 ±5.33
Delayed	16	3.0 ±0.14	2.9 ±0.14	33.3 ±6.33	33.8 ±5.29
EST x Feeding strategy					
Control x Early	8	2.7 ±0.21	2.9 ±0.22	27.8 ±7.47	37.5 ±7.65
Control x Delayed	8	3.2 ±0.19	2.8 ±0.21	40.6 ±8.86	30.0 ±7.25
Lower x Early	8	2.7 ±0.39	2.8 ±0.21	14.3 ±13.23	34.1 ±7.41
Lower x Delayed	8	2.8 ±0.21	3.0 ±0.19	24.0 ±8.54	37.5 ±7.65
P-values					
EST		0.44	0.40	0.88	0.98
Feeding strategy		0.25	0.30	0.94	0.66
EST x Feeding strategey		0.91	0.34	0.44	0.53
Sex		0.95	0.95	0.63	0.89

[1] Number of pens.
[2] Broilers with score of 3 or more were classified as dysbacteriosis positive.
[3] In total 100 and 161 broilers for d 0 and 14, respectively.

Natural antibodies

IgM NAb titer on the day of inoculation, d 6 PEI, and d 14 PEI neither showed an interaction between EST and feeding strategy (Table 5; $P \geq 0.14$), nor a main effect of EST ($P \geq 0.75$) or feeding strategy ($P \geq 0.74$). At d 6 PEI, males tended to show higher IgM NAb titer compared to females ($\Delta = 0.5$ titer; $P = 0.06$) and at d 14 PEI, IgM NAb titer was higher in males compared to females ($\Delta = 0.8$ titer; $P < 0.01$).

IgY NAb titer at d 6 PEI showed an interaction between EST and feeding strategy (Table 5; $P = 0.04$). Early feeding resulted in a higher IgY NAb titer compared to delayed feeding if incubated at lower EST ($\Delta = 0.6$ titer), but not if incubated at control EST. IgY NAb titer on the day of *Eimeria* inoculation and at d 14 PEI did not show an interaction between EST and feeding strategy ($P \geq 0.28$), nor a main effect of EST ($P \geq 0.41$) or feeding strategy ($P \geq 0.60$). There was no difference in IgY NAb titer between sexes at any dissection day ($P \geq 0.71$).

Table 5. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic day 17 – 19.5; 37.8°C (**control**) or 36.7°C (**lower**)) and/or feeding strategy after hatch (immediate access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on IgM and IgY natural antibody (NAb) titer against keyhole limpet hemocyanin of broilers at d 0, 6, or 14 post *Eimeria* inoculation (d 21 post hatch) to induce necrotic enteritis (LSmeans ± SEM).

		IgM			IgY		
	N[1]	day post *Eimeria* inoculation[2]			day post *Eimeria* inoculation[2]		
		0	6	14	0	6	14
EST							
Control	16	1.6	2.6	4.1	0.9	1.1	3.0
Lower	16	1.6	2.5	4.1	0.7	1.1	3.1
SEM		0.2	0.2	0.2	0.2	0.1	0.3
Feeding strategy							
Early	16	1.5	2.6	4.2	0.8	1.2	3.1
Delayed	16	1.7	2.6	4.1	0.8	1.1	3.1
SEM		0.3	0.2	0.2	0.2	0.1	0.3
EST x Feeding strategy							
Control x Early	8	1.6	2.3	4.4	1.0	1.0[ab]	3.0
Control x Delayed	8	1.5	3.0	3.9	0.8	1.3[ab]	3.1
Lower x Early	8	1.4	2.9	4.0	0.5	1.4[a]	3.1
Lower x Delayed	8	1.9	2.2	4.3	0.9	0.8[b]	3.0
SEM		0.4	0.3	0.3	0.2	0.2	0.4
P-values							
EST		0.99	0.75	0.93	0.41	0.71	0.90
Feeding strategy		0.91	0.99	0.74	0.60	0.42	0.98
EST x Feeding strategy		0.39	0.17	0.14	0.28	0.04	0.70
Sex		0.69	0.06	<0.01[3]	0.78	0.87	0.71

[a-b] Least squares means within a column and factor lacking a common superscript differ (P<0.05).
[1] Number of pens.
[2] In total 48, 51, and 57 broilers for d 0, 6, and 14, respectively.
[3] Titer 4.5 and 3.7 ±0.20 for males and females, respectively.

Footpad dermatitis

FPD incidence at d 6 PEI showed an interaction between sex and feeding strategy (Table 6; *P*=0.04). Early fed females had lower FPD incidence compared to delayed fed females (Δ=13.0%; data not shown), whilst early and delayed fed males did not differ in FPD incidence at this day. FPD indidence at d 6 PEI did not show an interaction between EST and feeding strategy (*P*=0.68), nor a main effect of EST (*P*=0.31). FPD incidence at d 7 PEI did not show an interaction between EST and feeding strategy (*P*=0.73), nor a main effect of feeding strategy (*P*=0.14). FPD incidence at d 7 PEI tended to be higher in control EST compared

to lower EST (Δ=15.5%; *P*=0.09). On the day of Eimeria inoculation and at d 14 PEI, FPD incidence did not show an interaction between EST and feeding strategy (*P*≥0.56), nor a main effect of EST (*P*≥0.13) or feeding strategy (*P*≥0.17). There was no difference in FPD incidence between sexes at the day of *Eimeria* inoculation, and at d 7 PEI or d 14 PEI (*P*≥0.20).

Table 6. Effect of eggshell temperature (**EST**) during late incubation (≥embryonic day 17 – 19.5; 37.8°C (**control**) or 36.7°C (**lower**)) and/or feeding strategy after hatch (immediate access to feed and water (**early**) or 51-54 h deprivation (**delayed**) on average footpad dermatitis (**FPD**) incidence of broilers at d 0, 6, 7, or 14 post *Eimeria* inoculation (d 21 post hatch) to induce necrotic enteritis (mean ±SE).

		FPD (%)[2]			
		day post *Eimeria* inoculation[3]			
	N[1]	**0**	**6**	**7**	**14**
EST					
Control	16	14.9 ±4.35	19.7 ±4.57	26.3 ±5.05	36.3 ±5.33
Lower	16	0.0 ±0.00	10.4 ±3.48	10.8 ±3.61	21.0 ±4.52
Feeding strategy					
Early	16	2.3 ±2.30	13.0 ±3.83	11.7 ±3.66	21.0 ±4.52
Delayed	16	16.1 ±4.91	17.1 ±4.32	26.0 ±5.14	36.3 ±5.33
EST x Feeding strategy					
Control x Early	8	2.8 ±2.74	18.4 ±6.29	18.4 ±6.29	30.0 ±7.25
Control x Delayed	8	29.0 ±8.15	21.1 ±6.61	34.2 ±7.70	42.5 ±7.75
Lower x Early	8	0.0 ±0.00	7.7 ±4.27	5.1 ±3.53	12.2 ±5.11
Lower x Delayed	8	0.0 ±0.00	13.2 ±5.48	17.1 ±6.37	30.0 ±7.25
P-values					
EST		0.98	0.31	0.09	0.13
Feeding strategy		0.99	0.65	0.14	0.17
EST x Feeding strategy		0.99	0.68	0.73	0.56
Sex		0.20	0.42	0.68	0.50
Body weight		0.48	0.82	0.32	0.11
Sex x Feeding strategy		n.a.	0.04	n.a.	n.a.

[1] Number of pens.
[2] Broilers with score of 1 or 2 were classified as FPD positive.
[3] In total 99, 153, 150, and 161 broilers for d 0, 6, 7, and 14, respectively.
differ (P<0.05).

DISCUSSION

The aim of this study was to investigate whether or not broiler resilience could be affected by EST during late incubation and/or by feeding strategy immediately post hatch. Resilience was defined in this study as the capability to absorb environmental disturbances and reorganize with minimal loss of function, according to Folke et al. (2010). Therefore, NE was induced at d 21 post hatch to model an environmental disturbance and loss of function was determined by measuring mortality and changes in BW. Disease morbidity measures were also determined, but as discussed in the following paragraphs, it is rather difficult to draw conclusions about resilience based on these morbidity measures.

Necrotic enteritis model

Regardless of treatments, the disturbance model successfully induced NE. After Eimeria inoculation, clinical symptoms of disease were observed and mortality and a severe reduction in ADG occurred. An overall NE incidence of 84.1% was found with NE lesion scores of 3.9 and 2.8 out of 6 at d 6 and 7 PEI respectively. Additionally, an increase of *E. Maxima* oocysts shed in the feces at d 8 PEI was seen. Remarkably, no increase in feces oocysts of *E. Acervulina* and *E. Mitis* was found, while both species were also inoculated. Likely, the predicted increase in oocysts shedding of these two species remained absent, because oocysts of both species were already found in feces prior to *Eimeria* inoculation. It was shown before that chickens can develop immunity to *Eimeria* (Jeurissen et al., 1996). However, although the presence of *E. Acervulina* and *E. Mitis* species prior to Eimeria inoculation may have affected the NE disease response, NE was still clearly induced successfully after inoculation of *Eimeria* at d 21.

The NE disease model induced mortality (17.5%) and a clear disturbance pattern in growth performance with a gradual drop in ADG up to 80 g during 3 to 4 days PEI. This makes the current NE disease model suitable to study broiler resilience, because loss of function can be determined through mortality rates and the speed and extension at which broilers dropped their BW. Remarkably, ADG sharply increased thereafter within 1 d back to pre-inoculation level. Because of this rapid recovery, it is hard to draw conclusions on possible differences in recovery speed. Recovery speed is also regarded as part of resilience, as 'reorganization' is part of the resilience definition (Folke et al., 2010). A different disease model that induces a disease with a slower recovery speed may give better insights in kinetics of reorganization after a disturbance. However, the rapid recovery in the current study may also indicate that broilers at this age are relatively resilient animals in terms of recovery speed. Results from the current study are representative for 4 wk old broilers of an old breeder flock and may help to manage NE, the most common disease in the poultry industry which occurs mainly around that age (Dahiya et al., 2006; Jones et al., 2019).

Interaction EST x feeding strategy

This study was the first study to investigate a possible interaction between prenatal thermal conditions and early feeding. It was hypothesized that EST and feeding strategy may interact with each other as EST may for instance affect embryo development in such a way that a newborn chick is better prepared for exogenous feed intake at hatch moment and consequently, the optimal combination of factors during neonatal life may affect broiler resilience in later life. The hypothesis was not confirmed as no interaction was found in the majority of the parameters. No difference in mortality, BW changes, or disease morbidity parameters were found with the exception of natural antibodies binding to keyhole limpet hemocyanin. At d 6 PEI, early fed broilers had a higher average NAb IgY titer compared to delayed fed broilers if these broilers were incubated at lower EST but not if they were incubated at control EST. At first instance the finding of only one significant interaction between EST and feeding strategy might not seem a very strong result, because the interaction was not consistently shown at other ages and occurred only in IgY but not in IgM isotype. However, the interaction was found at d 6 PEI at which the worst diseased state seemed to occur and may therefore only be manifested in a severely diseased state. The fact that differences were only found in IgY isotype and not IgM may be explained by the inflammatory immune responses that occur during NE, whereas Isotype IgY antibodies are strongly involved in the release of inflammatory mediators. So, the current finding of an interaction between EST and feeding strategy on NAb IgY titre at d 6 PEI is a minor indication that these two factors may interfere on a broilers immune system and as of yet, it is unknown whether or not EST during late incubation interacts with early moment of feeding post hatch. For future studies it might be interesting to investigate other EST patterns rather than the lower EST during late incubation from the current study. Prior to the current study we hypothesized that EST may perhaps affect embryo development in such a way that the neonatal chick could benefit optimally from immediate access to feed and water after hatch, for instance via altering intestinal morphology or digestive enzyme activities. However, the lower EST during late incubation in the current study may not have stimulated these parts of embryogenesis such that the hypothesized mechanism of interaction did not occur. This is supported by findings that a lower EST during late incubation impairs embryo development (Wijnen et al., 2020a - 2020b - 2022) instead of improving it (Wineland et al., 2001; Maatjens et al., 2016) or perhaps the only effect was a slower development of the embryo (Willemsen et al., 2010b).

EST

The current study showed that broiler resilience during later life appears to be modulated by EST during late incubation. Broilers incubated at a lower EST of 36.7°C during late incubation seemed to have impaired resilience, as they showed more difficulties coping with NE compared to broilers incubated at a control EST of 37.8°C. This impairment in resilience was especially shown by the ADG pattern. The first 4 days PEI, ADG was similar between

both EST groups and was not yet affected to a large extent by *Eimeria* inoculation. During the following 3 days thereafter, ADG was declining in both EST treatments, but the decline was larger in the lower EST group (from approximately 90 g/d to 10 g/d) compared to the control EST group (approx. 90 g/d to 40 g/d), with a significant difference at d 5 and d 8 and a tendency at d 6 PEI (P=0.06). Besides, the lower EST group reached their lowest ADG after *Eimeria* inoculation 1 d earlier and maintained at this low level 1 d longer compared to the control EST group. Lower EST incubated broilers also seemed to shed more oocysts in their feces, indicated by a higher *E. Maxima* OPG at d 8 PEI. To the author's knowledge, no other studies were published yet that investigated the effect of a lower incubation temperature during late incubation on broiler resilience. Nevertheless, current finding that EST might affect broiler resilience in later life is somewhat supported by two other studies, which found a higher mortality rate during the growing phase in broilers incubated at a lower late incubation temperature (Wijnen et al., 2020a; Jabbar et al., 2020).

Meanwhile, lower EST incubated broilers did not show a lower incidence or severity of morbidity parameters compared to control EST broilers. In fact, lower EST broilers had less severe clinical signs of coccidiosis at 2 wks PEI. This could be interpreted as a faster recovery of lower EST broilers, indicating improved resilience and this may appear contradictory to conclusions based on ADG. However, incidence and severity of morbidity can change rapidly over time and can vary even between consecutive days (Gholamiandehkordi et al., 2007; Timbermont et al., 2010). For instance, in the current study, NE lesion score was significantly higher on d 6 compared to d 7 PEI. It is possible that the development of morbidity over time varied between treatment groups. In such cases, conclusions about broiler resilience by looking at morbidity parameters at separate time points should be drawn with care, because it remains unclear what phase of functional loss was studied.

Feeding strategy

Early feeding tended (P=0.06) to lower total mortality after NE induction compared to delayed feeding (Δ=6.6%). Death can be regarded as the ultimate 'loss of function' as these broilers were unable to 'reorganize' after the disturbance. Therefore, this tendency is an indication that resilience in later life might be improved by early feeding.

Meanwhile, no difference was found between early and delayed fed broilers in the number of shed *Eimeria* oocysts and severity or incidence of NE lesions, coccidiosis, and dysbacteriosis. As discussed before, morbidity measures can provide useful information to study disease severity and incidence at specific timepoints. These morbidity measures may, however, be somewhat misleading when studying resilience, because pathogenesis can rapidly change over time and can vary significantly between consecutive days, meaning that essential information about loss of function can be missed if measurements are performed only at specific time points. A more convincing support of possible effects of feeding strategy on resilience was expected to be found in changes in BW after NE. Despite consistently higher

bodyweights of early fed broilers after onset of NE, the difference in BW between early and delayed fed broilers remained the same over time. Additionally, loss of ADG after onset of NE was similar between both feeding groups. This indicates that feeding strategy had no effect on the loss of function and therefore broiler resilience to NE in the 4th wk of life seems unaffected. Perhaps no differences in changes in BW were found because only surviving broilers were included in these analyses. The delayed feeding treatment tended to have higher mortality and likely dying broilers had the largest losses in BW. However, additional analysis on BW change, including the broilers that died, showed comparable results to those currently presented.

Feeding strategy might have an effect on broiler resilience that is more clearly pronounced during the first two to three wks post hatch. For instance, (Ao et al., 2012) also induced NE, but instead they inoculated *Eimeria* at d 9 post hatch and they showed that early fed broilers had increased resilience to NE in terms of growth performance. In the current study, NE was induced at d 21 post hatch and resilience was subsequently determined during the fourth wk of life. It could be that delayed fed broilers showed compensatory development, meaning that potential differences between early and delayed feeding strategy were not clearly pronounced in changes in BW. Studies showed that although full compensation was not observed, delayed fed broilers do indeed show compensatory BW gain to some extent (Juul-Madsen et al., 2004; Ao et al., 2012; Lamot et al., 2014; de Jong et al., 2017; Hollemans et al., 2018). Moreover, the majority of differences in immune response that were found between early and delayed fed broilers seems to diminish after 3 wks post hatch (Shira and Sklan, 2005; Simon et al., 2014; Hollemans et al., 2020). Hollemans et al. (Hollemans et al., 2020) also found no indication of different later life resilience between early and delayed fed broilers. In the latter study, early and 72 h delayed fed broilers of a prima breeder flock were challenged by housing them in poor sanitary conditions.

Early fed females had a lower incidence of FPD at d 6 PEI compared to delayed fed females (Δ=13.0%; P=0.04), whereas this difference was not found in males. Prevalence of FPD is predisposed by wet litter (Martland, 1985; Mayne, 2005) and wet litter can be a result of abnormal fecal droppings caused by intestinal problems. In such a case, a lower incidence of FPD in early fed females could be an indirect indicator of improved resilience. However, in the current study no indications of a difference in gut health between early and delayed fed broilers were found as coccidiosis and dysbacteriosis scores were similar between both treatments. Moreover, the difference in FPD was not very consistent as it was observed only on a single collection moment and only in females. Nevertheless, several other studies also found similar indications that early feeding, as factor of on-farm hatching, may reduce FPD as well as mortality rate during grow out (de Jong et al., 2019, 2020; Giersberg et al., 2021; Jessen et al., 2021). It should be noted though that effects of on farm hatching cannot only be attributed to early feeding but may be confounded with other factors such as transportation of eggs instead of day-old chicks or EST during the last days of incubation on the farm. Perhaps early feeding acts on FPD and mortality via a mechanism that is not expressed in BW changes. For instance,

early feeding may alter broiler activity levels, drinking behaviour, or nutritional needs, which all are factors that can also affect litter moisture content (Shepherd and Fairchild, 2010). Future studies could perhaps investigate which predisposing factor altered FPD incidence between early and delayed fed broilers and thereby gather new insights on possible later life effects of early feeding.

CONCLUSION

It can be concluded that EST during late incubation did not interact with feeding strategy on broiler resilience to NE at 4 wks of age in broilers of an old breeder flock. Indications were found that both a lower EST of 36.7°C during late incubation and 51-54 h delayed feeding each may impair later life resilience compared to control EST of 37.8°C and immediate access to feed and water after hatch. However, differences between treatments were not manifested consistently in the multiple parameters that were measured and therefore these conclusions are drawn with some restraint.

ACKNOWLEDGEMENT

The authors wish to thank Lagerwey hatchery for providing the eggs and HatchTech for funding the experiment. We would also like to thank caretakers and other team members from Poulpharm that assisted during the experiment, with special attention for Isaura Christiaens and Annelike Dedeurwaerder. We are also grateful for any assistance of WUR colleagues (Ger de Vries Reilingh, Ilona van den Anker-Hensen, Monique Ooms, Marcel Heetkamp, Tamme Zandstra), HatchTech colleague Thijme Hijink, and especially Wageningen University students Bart de Bont and Charlotte Visser.

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CHAPTER 6

BROILER RESILIENCE TO COLIBACILLOSIS IS AFFECTED BY INCUBATION TEMPERATURE AND POST-HATCH FEEDING STRATEGY

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Under review at Poultry Science

ABSTRACT

Colibacillosis is a poultry disease that negatively affects welfare and causes economic losses. Treatment with antibiotics raises concerns on antimicrobial resistance. Consequently, alternative approaches to enhance poultry resilience are needed. Access to feed and water directly after hatch (**early feeding**) may enhance resilience at later ages. Additionally, a high eggshell temperature (**EST**) during mid incubation may improve chick quality at hatch, supporting potential positive effects of early feeding. Effects of EST [37.8°C (**control**) or 38.9°C (**higher**)] during mid incubation (embryo days 7 – 14) and feeding strategy (early feeding or 48 hours **delayed feeding**) were tested in a 2 x 2 factorial arrangement. At hatch,~1800 broilers were divided over 36 pens and grown for 6 weeks. At day 8 post hatch, avian pathogenic *E. coli* (**APEC**) was inoculated intratracheally as model to investigate broiler resilience against respiratory diseases. Incidence and severity of colibacillosis, local infection, and systemic infection were assessed at 6 moments between 3 hours and 7 days post inoculation. Broilers were weighed daily during 13 days post inoculation and weekly thereafter. At higher EST, early feeding resulted in higher incidence of systemic infection compared to delayed feeding whereas at control EST, systemic infection was not different between feeding strategies. Regardless of EST, early compared to delayed feeding resulted in lower incidence of local infection, fewer BW deviations, and higher growth until day 35. In conclusion, early feeding could be considered as a strategy to enhance broiler resilience, but only when EST is not too high.

Keywords: broiler resilience, incubation, eggshell temperature, early feeding, delayed feeding, *Escherichia coli*, colibacillosis, APEC

INTRODUCTION

Colibacillosis is a common poultry disease caused by infection with avian pathogenic *Escherichia coli* (**APEC**). Poultry that suffer from a local APEC infection often show lesions in their respiratory tract and in case of a systemic infection, severe cardiac and hepatic lesions can occur (Gross, 1956). Consequently, poultry health and welfare are impaired and profit is reduced (Yogaratnam, 1995; Matthijs et al., 2017). Vaccination against this disease shows ambiguous results and treatment with antibiotics can contribute to the rise of antimicrobial resistant *E. coli* strains posing a serious threat for human health (van den Bogaard et al., 2001). Therefore, the poultry industry is searching for alternative approaches to cope with infectious diseases, like colibacillosis, for instance by enhancing animal resilience. Resilience can be defined as the capacity of an animal to deal with environmental disturbances and/or recover with minimal loss of function (Folke et al., 2010). In case the environmental disturbance concerns an infectious disease, a resilient broiler has a lower chance to become infected and, once it does get infected, it will show a milder drop in function (e.g. growth rate) and it will recover faster than a less-resilient broiler.

Resilience of poultry may be enhanced by optimizing perinatal conditions, such as the provision of feed and water directly after hatch, referred to as 'early feeding' (Roberts et al., 1928). In common practice, chicks have first access to feed and water upon arrival at the farm. Upon arrival, chicks are 36 to 48 hours of age or even older due to variation in hatch and pull time, processing, and transport duration (Careghi et al., 2005). Withholding chicks from feed and water during this period seems to result in suboptimal neonatal chick development, shown for instance by a loss in BW and impaired and/or delayed onset of immunocompetence compared to early fed chicks (Noy and Sklan, 1999; Shira et al., 2005; Lamot et al., 2014; Simon et al., 2014; Price et al., 2015; Wijnen et al., 2022). At later ages, early fed broilers showed enhanced growth performance, lower mortality rate, and different immune responses compared to delayed fed broilers (Juul-Madsen et al., 2004; Shira et al., 2005; Lamot et al., 2016; de Jong et al., 2017). Consequently, it can be speculated that early feeding may enhance disease resilience at later age as well. Some indications have been found that early fed broilers have higher resilience to intestinal diseases compared to delayed fed broilers (Dibner et al., 1998; Yi et al., 2005; Ao et al., 2012; Wijnen et al., 2021). However, studies investigating effects of post-hatch feeding strategy on disease resilience are limited and resilience to respiratory diseases, such as colibacillosis, have not been studied yet.

De Jong et al. (2017) concluded that effects of early feeding may be influenced by incubation temperature. Incubation temperature affects embryo development and consequently chick quality at hatch. Too high or too low incubation temperature can negatively affect gut morphology and digestive enzyme activity in newborn chicks (Wineland et al., 2006; Barri et al., 2011; Wijnen et al., 2020b) and it can be speculated that this might result in difficulties to digest and absorb first exogenous feed. Incubation temperature is most accurately reflected

by eggshell temperature (**EST**), which in turn reflects embryo body temperature (French, 1997). An increase or decrease in EST will result in an increase or decrease of embryonic metabolism, respectively, as embryos act poikilotherm during the major part of incubation (Romijn & Lonkhorst, 1955; Dietz & van Kampen, 1994; French, 1997; Lourens et al., 2006; Szdzuy et al., 2008). Currently, a constant EST of 37.8°C throughout incubation is considered to result in most optimal embryo development and chick quality at hatch (Lourens et al., 2005). However, Nangsuay et al. (2016) showed that an EST of 38.9°C from embryonic day (**E**) 7 onwards resulted in a higher yolk free body mass (**YFBM**) up to E16 compared to a constant 37.8°C EST. This may indicate enhanced embryo body development and chicks may hatch with higher YFBM, less residual yolk, and advanced development of organs, such as the gastrointestinal tract. Therefore, it can be hypothesized that a higher EST of 38.9°C during the second week of incubation advanced chick quality at hatch and that a better chick quality at hatch may enhance the proposed beneficial effects of early feeding on disease resilience.

This study evaluated whether or not broiler resilience to colibacillosis is affected by mid-incubation temperature, post-hatch feeding strategy, and their interaction. Two mid-incubation temperatures (high vs control) and two post-hatch feeding strategies (early vs delayed) were investigated with a two-by-two factorial design.

MATERIAL AND METHODS

An experiment was conducted between February and April 2021 at Wageningen University & Research, the Netherlands. All experimental procedures were approved by the Governmental Commission on Animal Experiments, The Hague, the Netherlands, approval number: 2018.W-0020.002.

Experimental design

The experiment was set up as a 2 x 2 factorial arrangement with EST during mid incubation and post-hatch feeding strategy as treatments. EST from E7 until E14 was set at 37.8°C (**control**) or at 38.9°C (**higher**). EST was 37.8°C for the remaining incubation periods for both EST treatments. Post-hatch feeding strategy included access to feed and water directly after hatch (**early feeding**) or 48 hours after hatch (**delayed feeding**).

Egg origin

A batch of hatching eggs from 1 house of a commercial 31-weeks-old Ross 308 broiler breeder flock were stored for 3 days at the broiler breeder farm (Boven-Leeuwen, the Netherlands) before transport to a commercial hatchery (Lagerwey BV, Lunteren, the Netherlands). Upon arrival at the hatchery, 10 egg trays with 150 eggs each were bulk weighed to determine the average egg weight of the batch (58.4 g) and 3 equal weight classes were made within 1.5 g of the average egg weight (56.9 - 57.9, 57.9 - 58.9, 58.9 - 59.9 g). Thereafter,

all eggs from the initial batch were weighed individually until 746 first-grade hatching eggs (clean, without hairline cracks or malformations) per weight class were selected (total 2,238 eggs). Eggs of each weight class were equally divided over 30 setter trays (type 150 Setter Tray, HatchTech, Veenendaal, the Netherlands) to exclude potential effects of initial egg weight on the study outcome (Wilson, 1991).

Incubation

All 30 trays were set in one incubator (PicoClimer setter HT-150, HatchTech, Veenendaal, the Netherlands) directly after indicated egg weighing procedures. Four EST sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK) were attached to the equator of the eggshell of 4 randomly chosen eggs, equally divided over the incubator, using silicone heat sink compound (Type 340; Dow Corning, Midland, MI) and a small piece (approx. 1.5 x 1.5 cm) of elastic permeable tape (Leukotape K, Essity, Hamburg, Germany). A 22-hour preincubation warming profile was applied (adapted from van Roovert-Reijrink et al., 2018) meaning that eggs were linearly warmed from room temperature to 27.8°C EST in 5 hours and from 27.8°C to 37.8°C EST in 17 hours. The moment that eggs reached an EST of 37.8°C was considered to be the start of incubation (E0). Until E7, incubator temperature was continuously adjusted, based on the median temperature of the 4 EST sensors to aim at an EST of 37.8°C. Relative humidity was maintained between 50 and 65%.

At E7, all setter trays were equally divided over 4 identical incubators (PicoClimer setter HT-150, HatchTech, Veenendaal, the Netherlands). Two incubators were set at an EST of 37.8°C (control EST), whereas the other 2 incubators were set at an EST of 38.9°C (higher EST). In the high EST treatment, EST setpoint was linearly increased over a 12 hour period. Until E14, EST control in all four incubators was performed as described above. Relative humidity was maintained between 40 and 55%.

At E14, all setter trays were collected from the 4 incubators and set in one incubator (PicoClimer setter HT-150, HatchTech, Veenendaal, the Netherlands). Until E17+17 hours, the EST was set at 37.8°C and controlled as described above. Relative humidity was maintained between 40 and 45%.

During E0 to E17+17 hours, eggs were turned every hour by an angle of 35° from horizontal and CO_2 levels were maintained below 3,500 ppm. At E17+17 hours, all eggs were candled. Clear eggs and eggs containing a dead embryo were opened to determine fertility. Eggs containing a viable embryo (95.8% of fertile eggs at set) were transferred to hatching baskets. Eggs were transferred to one hatcher basket per setter tray and all hatching baskets were set in one incubator (PicoClimer hatcher HT-150, HatchTech, Veenendaal, the Netherlands). Six EST sensors were attached to 6 randomly chosen eggs, equally divided over the incubator, and incubator air temperature set point was manually adjusted if the average of these 6 EST deviated from 37.8°C. After E19+12 hours, the incubator temperature was fixed at the actual setting, and EST was allowed to change as chicks started to emerge from

the eggshell. Relative humidity was maintained between 30 and 65% and CO_2 levels were maintained below 2,500 ppm.

Hatch and early feeding

From E19+12 hours onwards, every 3 hour the incubator was opened to check whether or not chicks had hatched. Any chick that hatched was marked with a colored dot on its head, using a permanent marker. After marking, chicks were placed back in their original hatcher basket to dry. Within 3 to 12 hours after a chick was marked, it was pulled from the incubator and classified either as a 2nd grade chick if any abnormality was observed (e.g. crossed beak, blindness, exposed brains, >2 legs, exposed yolk) or as a 1st grade chick (all remaining chicks). All 1st grade chicks were feather sexed, chick quality was determined (see data collection section below), labelled with a plasticized paper neck tag (size 5 x 2 cm), and transferred to hatcher baskets (HatchCare type, HatchTech, Veenendaal, the Netherlands). All baskets were stored in one section of a HatchCare unit (HatchTech Veenendaal, the Netherlands) until E21+13 hours. In half of these baskets (early feeding treatment), *ad libitum* starter diet (diet details provided in 'diet and vaccinations' section below) and fresh water were provided. The other half of the baskets had empty water gutters and feeding troughs (delayed feeding treatment). Relative humidity was maintained between 25 and 75% and CO_2 levels were maintained below 2,000 ppm. At E21+13 hours, all baskets were transported in a climate-controlled van designed for chick transportation (Chickliner, Renswoude, the Netherlands) for approximately 30 min to the grow out facilities (Wageningen University, Wageningen, the Netherlands).

Grow out

Layout. Upon arrival at the grow out facility (regarded as day 0), all 1st grade chicks were randomly allocated to 36 floor pens (9 replicate pens / treatment), accounting for the treatment and the sex (equal sex ratio within pen), resulting in 52 – 58 chicks per pen. Pens were located in 3 neighboring rooms (12 pens / room) and within each room, 3 blocks of 4 pens each were formed. Treatments were randomly allocated within a block. Pen size was 200 x 100 cm, contained 7 drinking nipples with drip cups and had one feeder pan. The concrete pen floor was covered with a 1-cm-thick layer of wood shavings. Broilers were grown for 6 weeks.

Delayed feeding. Pens from the delayed feeding treatment were temporarily divided with hardboard into an unfed side and a fed side. Furthermore, in the delayed fed pens, all drinking nipples were temporarily removed and a stand-alone drinker was provided in the fed side. Sides were out of sight from each other. All broilers belonging to the delayed feeding groups were first placed in the unfed side of the pen and each broiler was relocated individually to the fed side of the pen between 48 - 54 hours after it had hatched. All broilers within the early fed pens had access to the entire pen from placement onwards. Additionally, at placement, the

wood shavings of all 36 pens were covered with cardboard to prevent any litter consumption in the delayed feeding treatment. Hardboard (delayed fed pens) and cardboard (all pens) were removed once the last broiler was relocated to the fed side and stand-alone drinkers were exchanged for drinking nipples (delayed fed pens). Pen treatment was blinded from that moment onwards.

Diet and vaccinations. Once fed, feed and water were provided *ad libitum*. A starter diet (ME broiler = 2,850 kcal/kg, CP = 216.5 g/kg, digestible lysine = 11.01 g/kg) was provided from hatch until day 10, a grower diet (ME broiler = 2,950 kcal/kg, CP = 201.3 g/kg, digestible Lysine = 10.25 g/kg) from day 10 until 24, and a finisher diet (ME broiler = 2,999 kcal/kg, CP = 188.8 g/ kg, digestible Lysine = 9.51 g/kg) was provided from day 24 onwards. All diets were pelleted with a diameter 3.0 - 3.3 mm, did not contain coccidiostats, and were produced by Research Diet Services (RDS, Wijk bij Duurstede, the Netherlands) according to the guidelines of the Federation Dutch Animal Feed chain (CVB, 2016). Broilers were not vaccinated at the hatchery. At day 29, infectious bronchitis vaccine (Nobilis IB Ma5, batch A267B1J01, MSD) buffered in sterile blue coloured solvent (batch G554A02, MSD) was administered via a droplet in one eye and a droplet in one nostril.

Housing. Ambient temperature setpoint was 28°C at placement and was linearly decreased to 18°C at day 27, and this setpoint was maintained until the end of the study. Early and delayed fed broilers can differ in their preferred ambient temperature (Wijnen et al., 2022), and consequently, a heat lamp (100W infrared incandescent PAR38) was provided at approx. 15 cm above broiler height (adjusted manually with age) in the center of each pen, meaning that each broiler could choose its own preferred ambient temperature. Relative humidity setpoint was 60 – 70 % up to 3 days of age and 50 – 60 % thereafter. Continuous light was provided until day 2, and from day 2 onwards, 1 hour of darkness was added each day until a light schedule of 6 hours darkness : 18 hours light was provided by day 7 and onwards.

Disease model

Clinical respiratory colibacillosis was induced by adapting a disease model from Ask et al. (2006a). At day 8, 34 broilers of both sexes (equal ratio) were randomly selected within each pen for *E. coli* inoculation whereas the remaining broilers within each pen (n = 18 - 24) were selected for placebo inoculation. Broilers were inoculated intratracheally with either 0,3 mL phosphate buffered saline (**PBS**) for the placebo broilers, or 0,3 mL PBS containing avian pathogenic *Escherichia coli* serotype O78:K80 strain 506 (**APEC**) to induce colibacillosis. The inoculation was performed using a blunted anal cannula fitted on a 1.0 mL syringe. The APEC originated from a frozen culture (-70°C) that had previously been isolated from an inflamed pericardium of a commercial broiler suffering from natural colibacillosis (Van Eck & Goren, 1991). The inoculum was prepared as described by Matthijs et al. (2003), which resulted in 1.53 x 10^7 CFU/mL (determined by the Veterinarian Microbiological Diagnostic Centre,

Utrecht University, Utrecht, the Netherlands). All intratracheal inoculations were performed by trained personnel.

Data collection

Incubation duration was calculated as the number of hours from E0 (start of incubation) to emergence from the eggshell. Chick quality from all 1st grade chicks was determined by measuring BW, chick length from beak-tip to toe-tip, and navel score according to the protocol of Reijrink et al. (2009). Additionally, every 36th chick per EST treatment that hatched was euthanized through decapitation until 25 chicks per EST treatment were collected. Residual yolk (**RY**) and heart were weighed on a three-decimal scale and YFBM was calculated as BW minus RY weight. Relative heart weight was calculated as heart weight divided by YFBM times 100.

Disease morbidity was assessed by observation of lesions at 6 time points post inoculation (**p.i.**) (3 hours, 12 hours, 1 day, 2 days, 4 days, and 7 days). At each time point, 2 APEC inoculated broilers per pen (1 female and 1 male) were randomly selected for dissection. At 7 days p.i., 8 additional APEC inoculated broilers per pen (equal sex ratio) were randomly selected for dissection. At each time point, the selected broilers were euthanized by decapitation and the presence of lesions in left and right thoracic air sac, pericardium, and serosal surface of the liver were macroscopically assessed. Lesions of each organ were scored 0 (no lesions), 0.5 (a single pinhead-sized inflammatory spot), 1 (two or more pinhead-sized spots), 2 (fibrinous patches on various locations), or 3 (extensive fibrination and exudation) according to the protocol of Van Eck and Goren (1991) by trained poultry veterinarians. The sex of all dissected birds was verified by checking the gonads.

Subsequently, incidences of total lesions, local lesions, and systemic lesions were determined as follows. For incidence of total lesions, all four lesion scores were summed and if the sum was > 0, the broiler was classified as colibacillosis positive. For incidence of local lesions, both air sac lesion scores were summed and in if the sum > 0, the broiler was classified local lesions positive. For incidence of system lesions, lesion scores of the pericardium and liver were summed and if the sum > 0, the broiler was classified systemic lesions positive.

Severity of lesions was evaluated by calculating total mean lesion score (**tMLS**), local mean lesion score (**lMLS**), and systemic mean lesions score (**sMLS**) as follows. tMLS was calculated for each broiler that was classified colibacillosis positive by the sum of all four lesion scores. lMLS was calculated for each broiler that was classified local lesions positive by the sum of both air sac lesion scores. sMLS was calculated for each broiler that was classified systemic lesions positive by the sum of pericardium lesion score and liver lesion score. Severity classifications will be explained in the statistical analysis section.

The presence of *E. coli* in air sacs (local infection) and blood (systemic infection) was determined in 2 broilers / pen for each time point, randomly selected among the dissected animals, according to an adapted protocol from Cuperus et al. (2016). For isolation of *E. coli*

from blood, right before decapitation a part of the skin above the wing vein was disinfected and approximately 1 mL of blood was collected with single use needles and syringes and without any anticoagulant. Immediately thereafter, three droplets of blood were dripped on a McConkey agar plate (Balis, Boven-Leeuwen, the Netherlands) and spread with a disposable spreader, using the spread plate technique. For isolation of *E. coli* from air sacs, the thoracic air sac with the most severe lesions was swabbed immediately after postmortem examination using transport swabs with Amies medium (Uni-ter CLR 230397 lot 30380, Meus, Piove di Sacco, Italy). The left air sac was swabbed in case lesion severity was similar between left and right air sac or in case no lesions occurred. Swabs with Amies medium were stored in a fridge (~ 7°C) until the next day and then spread on McConkey agar using a streak technique. Bacterial growth was evaluated after overnight incubation at 37.5°C by counting the number of colony-forming units (**CFU**). Incidence of *E. coli* in air sacs and blood was calculated by classifying agar plates with > 0 CFU as *E. coli* positive. Amount of *E. coli* was determined by counting the number of CFU for each agar plate that was classified as *E. coli* positive.

All broilers were individually weighed daily during 13 days p.i., except at 7 days and 12 days p.i.. Broilers that died or were dissected during these 13 days were only included in analysis if BW could be recorded for at least 5 consecutive days during these 13 days p.i. (n = 806 broilers). For each day p.i., standardized BW deviation was determined by comparing the standardized BW from each APEC inoculated broiler to the average BW of placebo inoculated broilers from the same sex and treatment. 'Standardized BW' was BW that was standardized to standardized deviation of corresponding treatment group and day p.i. to correct for scaling differences in standardized deviations between ages and treatment groups. The natural logarithm of the variance (**LNvar**), skewness, and lag-one autocorrelation of standardized BW deviations were calculated as resilience indicators according to a protocol of Berghof et al. (2019a). Additionally, all broilers were weighed individually every week to observe growth performance until the end of the experiment (day 42).

Survival was monitored during 34 days p.i.. During the first 3 days p.i., mortality was checked every 3 hours. During the remaining period, mortality was checked daily. Broilers were culled if a humane endpoint was reached as described by Berghof et al. (2019b). Moment of death or cull was noted, and carcasses were saved in a freezer for necropsy at the end of the experiment. Necropsy was performed by a poultry veterinarian to determine suspected cause of death. Causes of death were divided into colibacillosis (septic hemorrhage in organs or lesions in air sacs, pericardium, and/or liver) or other reasons than the *E. coli* infection.

Statistical analyses

All data were analyzed using the statistical software package SAS (Version 9.4, SAS Institute, 2010). A *P*-value < 0.05 was considered to be significant, and *P*-values >0.05 and <0.10 were considered to be a tendency. The model used for all data at hatch was

$$Yij = \mu + ESTi + SEXj + EST \times SEXij + eij \quad (1)$$

where Y_{ij} =the dependent variable, μ=the overall mean, EST_i = eggshell temperature during mid incubation ($_i$ = 37.8 or 38.9°C), SEX_j = sex ($_j$ = female or male), $EST \times SEX_{ij}$ = the interaction between EST and SEX, and e_{ij} = the error term. Hatching basket was considered to be the experimental unit by adding hatching basket nested within incubator as a random factor. The EST × SEX interactions was excluded from the model when $P > 0.05$. The PROC MIXED procedure was used to analyze incubation duration, BW, RY, YFBM, chick length and relative heart weight. Model assumptions were verified by inspection of 'raw residuals vs predicted values' plot and 'Q-Q' plot of the residuals and skewness and kurtosis between -2 to +2. All data were normally distributed and presented as LSmeans ± SEM. The PROC GLIMMIX procedure was used to analyze navel score, using a multinomial distribution, and a cumlogit link function. Navel score is presented as mean ± SE.

The basic model used for all post hatch data was

$$Y_{ijk} = \mu + EST_i + FEED_j + EST \times FEED_{ij} + SEX_k + EST \times SEX_{ik} + FEED \times SEX_{jk} + EST \times FEED \times SEX_{ijk} + e_{ijk} \quad (2)$$

where Y_{ijk} = the dependent variable, μ = the overall mean, EST_i = eggshell temperature during mid incubation ($_i$ = 37.8 or 38.9°C), $FEED_j$ = feeding strategy ($_j$ = early or delayed), $EST \times FEED_{ij}$ = the interaction between EST and FEED, SEX_k = sex ($_k$ = female or male), $EST \times SEX_{ik}$ = the interaction between EST and SEX, $FEED \times SEX_{jk}$ = the interaction between FEED and SEX, $EST \times FEED \times SEX_{ijk}$ = the 3-way interaction between EST and FEED and SEX, and e_{ijk} = the error term. Pen was considered to be the experimental unit by adding pen (1 – 36) nested within block (1 – 9) as a random factor. Treatment × SEX interactions were excluded from the model when $P > 0.05$.

The PROC GLIMMIX procedure was used to analyze incidence of colibacillosis, incidence of local lesions, incidence of systemic lesions, incidence of *E. coli* in air sacs, incidence of *E. coli* in blood, tMLS, lMLS, sMLS, amount of *E. coli* in air sacs, and amount of *E. coli* in blood. tMLS was divided into 6 equal classes (0.5 - 2 = class 1, 2.5 – 4 = class 2, 4.5 – 6 = class 3, 6.5 – 8 = class 4, 8.5 – 10 = class 5, >10 = class 6), lMLS into 5 equal classes (0.5 – 1 = class 1, 1.5 – 2 = class 2, 2.5 – 3 = class 3, 3.5 – 4 = class 4, >4 = class 5), and sMLS into 5 equal classes (0.5 – 1 = class 1, 1.5 – 2 = class 2, 2.5 – 3 = class 3, 3.5 – 4 = class 4, >4 = class 5). Dissection moment was added to model 2 as a fixed factor. All incidences were analyzed with a binary distribution and a logit link function in model 2. tMLS, lMLS, and sMLS were analyzed with a multinomial distribution and a cumulative logit link function in model 2. Amount of *E. coli* in air sacs and *E. coli* in blood were analyzed with a Poisson log link function in model 2 with dissection moment added as a fixed factor. Data are expressed as mean ± SE.

The PROC MIXED procedure was used to analyze weekly BW (separately for each week) and LNvar, skewness, and lag-one autocorrelation of standardized BW deviations. Model assumptions were verified as previously indicated. All data were normally distributed. Data are expressed as LSmeans ± SEM.

The PROC PHREG procedure (cox proportional hazard model) was used to perform a survival analysis on APEC inoculated broilers for the period p.i. (> 7 days). Broilers that were dissected or euthanized at the end of the experiment were censored. Broilers that were suspected during necropsy to have died or culled due to other reason than APEC were excluded from analysis (n = 10). Block was added as random factor. Model assumptions were verified by a supremum test (Kleinbaum and Klein, 2012).

RESULTS

Chick quality at hatch

Chick length at hatch showed an interaction between EST × sex. Control and higher EST females did not differ in length, whereas control EST males were shorter than higher EST males (Δ = 0.1 cm; $P = 0.02$; Table 1). Higher EST had shorter incubation duration compared to control EST (Δ = 4 hours; $P < 0.0001$). Chick BW, RY, YFBM, relative heart weight, and navel score did not differ between EST groups ($P \geq 0.26$).

Table 1. Effect of eggshell temperature (**EST**) during mid incubation[1] on incubation duration and chick quality characteristics at hatch moment.

	n[2]	Duration (h)	Chick weight (g)	Residual yolk weight (g)	Yolk-free body mass (g)	Chick length[3] (cm)	Navel condition[4] (score)	Heart[5] weight (%)
EST								
Control	15	487[a]	43.02	5.81	37.33	18.6	1.5 ± 0.02	0.82
Higher	15	483[b]	43.10	5.81	37.05	18.7	1.5 ± 0.02	0.81
SEM		0.4	0.052	0.237	0.251	0.02	-	0.022
P-values								
EST		<0.0001	0.26	0.99	0.45	<0.01	0.92	0.83
EST × Sex		-	-	-	-	0.02[6]	-	-

Note: data are presented as least square mean ± SEM, except for navel condition (mean ± SE).

[1] EST during mid incubation (embryo days 7 – 14) was either 37.8°C (**Control**) or 38.9°C (**Higher**), and the remaining incubation period EST was 37.8°C for both treatment groups.

[2] Trays nested in incubator.

[3] Body length from beak-tip to toe-tip.

[4] Navel condition: score 1 (perfect), 2 (discolored/opened < 2 mm), 3 (discolored/opened > 2 mm).

[5] Weight relative to yolk-free body mass.

[6] Length was 18.7[ab], 18.6[c], 18.8[a], and 18.7[b] cm for control females, control males, higher females, higher males respectively.

[a-b] Least square means within a column lacking a common superscript differ ($P < 0.05$).

Colibacillosis

Incidence of colibacillosis did not show an interaction between EST and feeding strategy ($P = 0.67$; Table 2), nor a main effect of EST ($P = 0.26$). Incidence of colibacillosis tended to be lower for early fed broilers compared to delayed fed broilers ($\Delta = 2$ %; $P = 0.09$). tMLS showed an interaction between EST and feeding strategy ($P < 0.01$). At control EST, tMLS was higher in delayed fed broilers than early fed broilers ($\Delta = 0.8$ lesion score), whereas the opposite was found at higher EST ($\Delta = 0.7$ lesion score).

Table 2. Effect of eggshell temperature (**EST**) during mid incubation[1] and post-hatch feeding strategy[2] on incidence and severity of colibacillosis lesions[3] in broilers during colibacillosis[4].

	n[5]	No lesions[6] (%)	tMLS[7] (score)
EST			
Control	18	38 ± 2.6	3.0 ± 0.25
Higher	18	37 ± 2.5	2.9 ± 0.23
Feeding strategy			
Delayed	18	37 ± 2.5	2.9 ± 0.24
Early	18	39 ± 2.6	2.9 ± 0.24
EST × Feeding strategy			
Control × Delayed	9	34 ± 3.6	3.4 ± 0.37[a]
Control × Early	9	41 ± 3.7	2.6 ± 0.32[b]
Higher × Delayed	9	39 ± 3.5	2.5 ± 0.29[b]
Higher × Early	9	36 ± 3.6	3.2 ± 0.34[a]
P-values			
EST		0.26	0.90
Feeding strategy		0.09	0.77
EST × Feeding strategy		0.67	<0.01

Note: data are presented as mean ± SE.

[1] EST during mid incubation (embryo days 7 – 14) was either 37.8°C (**Control**) or 38.9°C (**Higher**), and the remaining incubation period EST was 37.8°C for both treatment groups.

[2] Feeding strategy was either direct access to feed and water after hatch (**Early**) or 48 hours after hatch (**Delayed**).

[3] Sum of lesion scores from left air sac, right air sac, pericardium, and liver, each scored 0 (clean), 0.5 (single spot), 1 (two or more spots), 2 (patches), or 3 (extensive fibrination).

[4] Colibacillosis was induced by intratracheal *E. coli* (O78:K80 strain 506) inoculation at day 8 of age (dose 0.3 mL of 1.53 x 10^7 CFU/mL).

[5] Pens, with 20 broilers dissected / pen, divided over 6 moments post inoculation (3 & 12 hours, 1-2-4-7 days).

[6] Sum of lesion score[3] = 0.

[7] tMLS = total Mean Lesion Score from broilers with sum of lesions score[3] > 0.

[a-b] Means within a column and factor lacking a common superscript differ ($P < 0.05$).

Local *E. coli* infection

Incidence and amount of *E. coli* in air sacs did not show an interaction between EST and feeding strategy ($P \geq 0.31$; Table 3), nor a main effect of EST ($P \geq 0.18$) or feeding strategy ($P \geq 0.57$). Incidence of local lesions did not show an interaction between EST and feeding strategy ($P = 0.55$), nor a main effect of EST ($P = 0.20$). Incidence of local lesions was lower for early fed broilers compared to delayed fed broilers ($\Delta = 6$ %; $P = 0.046$). lMLS did not show an interaction between EST and feeding strategy ($P = 0.24$), nor a main effect of EST ($P = 0.93$) or feeding strategy ($P = 0.42$).

Table 3. Effect of eggshell temperature (**EST**) during mid incubation[1] and post-hatch feeding strategy[2] on incidence and severity of *E. coli* in air sac[3] and local lesions[4] in broilers during colibacillosis[5].

	n[6]	E. *coli* in air sac		Local lesions	
		Incidence[7] (%)	Amount[8] (CFU)	Incidence[9] (%)	Severity[10] (lMLS)
EST					
Control	18	34 ± 3.2	121 ± 81.2	61 ± 2.6	1.9 ± 0.12
Higher	18	41 ± 3.3	100 ± 62.2	65 ± 2.5	1.9 ± 0.12
Feeding strategy					
Delayed	18	39 ± 3.3	132 ± 75.1	66 ± 2.5[a]	1.9 ± 0.12
Early	18	36 ± 3.3	88 ± 65.2	60 ± 2.6[b]	1.9 ± 0.12
EST × Feeding strategy					
Control × Delayed	9	38 ± 4.6	159 ± 120.3	65 ± 3.6	2.1 ± 0.18
Control × Early	9	30 ± 4.5	81 ± 101.8	56 ± 3.7	1.7 ± 0.17
Higher × Delayed	9	40 ± 4.7	104 ± 91.2	67 ± 3.5	1.8 ± 0.15
Higher × Early	9	42 ± 4.8	95 ± 85.8	63 ± 3.6	2.0 ± 0.18
P-values					
EST		0.18	0.37	0.20	0.93
Feeding strategy		0.57	0.94	0.05	0.42
EST × Feeding strategy		0.31	0.55	0.55	0.24

Note: data are presented as mean ± SE.

[1] EST during mid incubation (embryo days 7 – 14) was either 37.8°C (**Control**) or 38.9°C (**Higher**), and the remaining incubation period EST was 37.8°C for both treatment groups.

[2] Feeding strategy was either direct access to feed and water after hatch (**Early**) or 48 hours after hatch (**Delayed**).

[3] *E. coli* colony forming units (**CFU**) from air sac swab plated on McConkey-agar.

[4] Sum of lesion scores from left and right air sac, each scored 0 (clean), 0.5 (single spot), 1 (two or more spots), 2 (patches), or 3 (extensive fibrination).

[5] Colibacillosis was induced by intratracheal *E. coli* (O78:K80 strain 506) inoculation at day 8 of age (dose 0.3 mL of 1.53 x 10^7 CFU/mL).

[6] Pens, with 20 broilers dissected / pen, divided over 6 moments post inoculation (3 & 12 hours, 1-2-4-7 days).

[7] Classified 'Incidence positive' if CFU[3] > 0.

[8] From *E. coli* in airsac incidence positive broilers[7].

[9] Classified 'Incidence positive' if local lesion score[4] >0.

[10] lMLS = local Mean Lesion Score from local lesion incidence positive broilers[9].

[a-b] Means within a column and factor lacking a common superscript differ ($P < 0.05$).

Systemic *E. coli* infection

Incidence of *E. coli* in blood ($P = 0.01$) and incidence of systemic lesions ($P = 0.03$) both showed and interaction between EST and feeding strategy (Table 4). At control EST, no effect of feeding strategy was found, but at higher EST, delayed fed broilers had a lower incidence of *E. coli* in blood and a lower incidence of systemic lesions than early fed broilers ($\Delta = 11\%$ and $\Delta = 10\%$, for *E. coli* in blood and systemic lesions, respectively). Amount of *E. coli* in blood did not show an interaction between EST and feeding strategy ($P = 0.99$), nor a main effect of EST ($P = 0.71$) or feeding strategy ($P = 0.49$). sMLS did not show an interaction between EST and feeding strategy ($P = 0.45$), nor a main effect of EST ($P = 0.83$) or feeding strategy ($P = 0.67$).

Table 4. Effect of eggshell temperature (**EST**) during mid incubation[1] and post-hatch feeding strategy[2] on incidence and severity of *E. coli* in blood[3] and systemic lesions[4] in broilers during colibacillosis[5].

	n[6]	E. *coli* in blood		Systemic lesions	
		Incidence[7] (%)	Amount[8] (CFU)	Incidence[9] (%)	Severity[10] (sMLS)
EST					
Control	18	20 ± 2.7	10 ± 16.9	28 ± 2.4	1.1 ± 0.24
Higher	18	18 ± 2.6	17 ± 50.7	27 ± 2.4	1.0 ± 0.22
Feeding strategy					
Delayed	18	18 ± 2.6	16 ± 51.5	26 ± 2.3	1.0 ± 0.25
Early	18	20 ± 2.8	11 ± 14.9	29 ± 2.4	1.1 ± 0.21
EST × Feeding strategy					
Control × Delayed	9	23 ± 4.0[a]	13 ± 24.3	30 ± 3.4[ab]	1.3 ± 0.34
Control × Early	9	17 ± 4.0[ab]	8 ± 22.8	26 ± 3.3[ab]	1.0 ± 0.32
Higher × Delayed	9	13 ± 3.2[b]	20 ± 141.9	22 ± 3.1[b]	0.8 ± 0.36
Higher × Early	9	24 ± 4.1[a]	14 ± 19.9	32 ± 3.5[a]	1.3 ± 0.28
P-values					
EST		0.54	0.71	0.89	0.83
Feeding strategy		0.42	0.49	0.38	0.67
EST × Feeding strategy		0.01	0.99	0.03	0.45

Note: data are presented as mean ± SE.

[1] EST during mid incubation (embryo days 7 – 14) was either 37.8°C (Control) or 38.9°C (Higher), and the remaining incubation period EST was 37.8°C for both treatment groups.

[2] Feeding strategy was either direct access to feed and water after hatch (Early) or 48 hours after hatch (Delayed).

[3] *E. coli* colony forming units (CFU) from blood swab plated on McConkey-agar.

[4] Sum of lesion scores from pericardium and liver, each scored 0 (clean), 0.5 (single spot), 1 (two or more spots), 2 (patches), or 3 (extensive fibrination).

[5] Colibacillosis was induced by intratracheal *E. coli* (O78:K80 strain 506) inoculation at day 8 of age (dose 0.3 mL of 1.53 x 10^7 CFU/mL).

[6] Pens, with 20 broilers dissected / pen, divided over 6 moments post inoculation (3 & 12 hours, 1-2-4-7 days).

[7] Classified 'Incidence positive' if CFU[3] ≥1.

[8] From *E. coli* in blood incidence positive broilers[7].

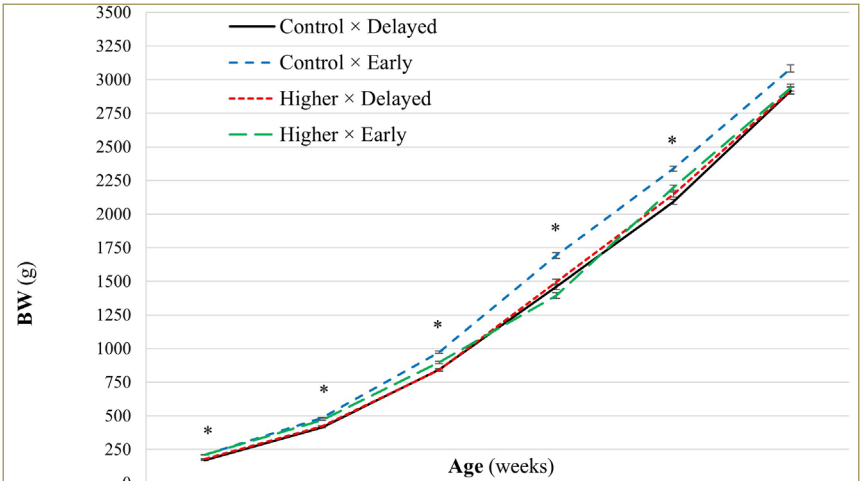
[9] Classified 'Incidence positive' if systemic lesion score[4] >0.

[10] sMLS = systemic Mean Lesion Score from systemic lesion incidence positive broilers[9].

[a-b] Means within a column and factor lacking a common superscript differ ($P < 0.05$).

Body weight

Body weights showed an interaction between EST and feeding strategy at all weeks measurements ($P \leq 0.03$; Figure 1), except for week 6 ($P = 0.17$). At week 1, one day prior to inoculation, early fed broilers incubated at higher EST or control EST had highest BW, delayed fed broilers incubated at higher EST had intermediate BW, and delayed fed broilers incubated at control EST had lowest BW. At week 2 and 3, early fed broilers incubated at control EST had highest BW, early fed broilers incubated at higher EST had intermediate BW, and delayed fed broilers incubated either at control EST or higher EST had lowest BW. At week 4 and 5, early fed broilers incubated at control EST had higher BW compared to the other treatment groups which were similar to each other. At week 6, early fed broilers tended to have higher BW compared to delayed fed broilers ($\Delta = 92$ g; $P = 0.08$;). EST had no effect on BW at week 6 ($P = 0.19$).



	1	2	3	4	5	6
Control × Delayed	171 ^{c}	414 ^{c}	842 ^{c}	1460 ^{b}	2090 ^{b}	2917
Control × Early	211 ^{a}	486 ^{a}	973 ^{a}	1692 ^{a}	2337 ^{a}	3082
Higher × Delayed	179 ^{b}	423 ^{c}	840 ^{c}	1493 ^{b}	2145 ^{b}	2921
Higher × Early	210 ^{a}	468 ^{b}	897 ^{b}	1395 ^{b}	2196 ^{b}	2940

Figure 1. Effect of the interaction between eggshell temperature (37.8°C (**Control**) or 38.9°C (**Higher**)) during mid incubation (embryo days 7 – 14) with post-hatch feeding strategy (direct access to feed and water after hatch (**Early**) or 48 hours deprivation (**Delayed**)) on broiler BW weekly post avian pathogenic *E. coli* inoculation performed at day 8. Data are presented as LSMean. Error bars indicate SEM. * indicates significant interaction between EST and feeding strategy. ^{abc} indicates least square means within a week lacking a common superscript differ significant. Significant = $P < 0.05$.

LNvar, skewness, and lag-one autocorrelation of standardized BW deviations during 13 days p.i. did not show an interaction between EST and feeding strategy ($P \geq 0.12$; Table 5), nor a main effect of EST ($P \geq 0.19$). LNvar was lower for early fed broilers compared to delayed fed broilers ($\Delta = 0.27$; $P = 0.02$), whereas skewness and lag-one autocorrelation did not differ between feeding strategies ($P \geq 0.19$).

Table 5. Effect of eggshell temperature (**EST**) during mid incubation[1] and post-hatch feeding strategy[2] on standardized deviations of BW[3] from broilers during 13 days post inducing colibacillosis[4].

		Standardized BW deviations		
	n[5]	**LNvar**	**Skewness**	**Lag-one autocorrelation**
EST				
Control	18	-2.55	-0.31	0.32
Higher	18	-2.70	-0.28	0.30
SEM		0.081	0.04	0.013
Feeding strategy				
Delayed	18	-2.49[a]	-0.33	0.31
Early	18	-2.76[b]	-0.25	0.31
SEM		0.082	0.04	0.013
EST × Feeding strategy				
Control × Delayed	9	-2.51	-0.36	0.31
Control × Early	9	-2.59	-0.25	0.33
Higher × Delayed	9	-2.48	-0.30	0.30
Higher × Early	9	-2.92	-0.26	0.30
SEM		0.12	0.06	0.019
P-values				
EST		0.19	0.67	0.35
Feeding strategy		0.02	0.19	0.79
EST × Feeding strategy		0.12	0.57	0.64

Note: data are presented as least square mean ± SEM.
[1] EST during mid incubation (embryo days 7 – 14) was either 37.8°C (**Control**) or 38.9°C (**Higher**), and the remaining incubation period EST was 37.8°C for both treatment groups.
[2] Feeding strategy was either direct access to feed and water after hatch (**Early**) or 48 hours after hatch (**Delayed**).
[3] Deviation of BW compared to placebo. Standardized and calculated as provided in Material and Methods section.
[4] Colibacillosis was induced by intratracheal *E. coli* (O78:K80 strain 506) inoculation at day 8 of age (dose 0.3 mL of 1.53 x 10^7 CFU/mL).
[5] Pens (approx. 34 broilers observed / pen)
[a-b] Least square means within a column and factor lacking a common superscript differ ($P < 0.05$).

Survival

First week mortality was low and similar between treatments (total 0.6 %). Survival probability p.i. did not show an interaction between EST and feeding strategy ($P = 0.10$; Figure 2C), nor a main effect of feeding strategy ($P = 0.68$; Figure 2B). Survival probability was higher for broilers incubated at control EST compared to higher EST (Δ = 4 %; $P = 0.04$; Figure 2A).

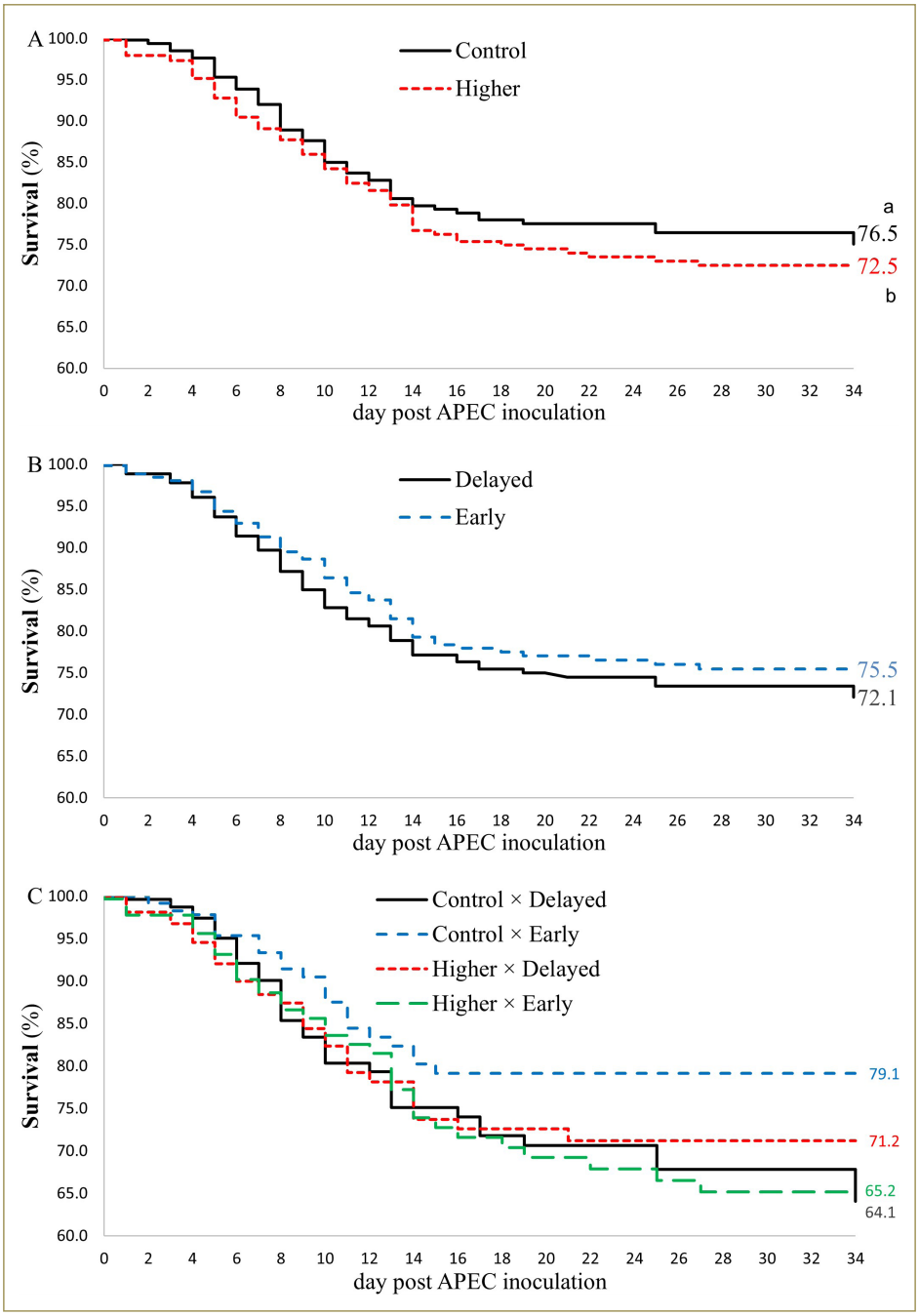


Figure 2. Effect of (**A**) eggshell temperature (37.8°C (**Control**) or 38.9°C (**Higher**)) during mid incubation (embryo days 7 – 14) and (**B**) post-hatch feeding strategy (direct access to feed and water after hatch (**Early**) or 48 hours deprivation (**Delayed**)) and (C) interaction between incubation temperature and post hatch feeding strategy on broiler survival probability during 34 days post avian pathogenic *E. coli* (**APEC**) inoculation at day 8 post hatch. [a-b] survival probabilities differ ($P < 0.05$).

DISCUSSION

We hypothesized that a higher EST of 38.9°C during mid incubation would accelerate embryo development, improve chick quality at hatch, and consequently enhance the proposed positive effect of early feeding on resilience to colibacillosis more than a control EST of 37.8°C would do. Our findings showed that systemic *E. coli* infection (pericardium and liver) indeed showed an interaction between EST and post-hatch feeding strategy, but opposite to what was expected, whereas local *E. coli* infection (air sacs) was only affected by post-hatch feeding strategy.

A higher incidence of systemic *E. coli* infection was found in early fed broilers compared to delayed fed broilers when incubated at higher EST, whereas at control EST systemic *E. coli* infection was not different between feeding strategies. This finding was expressed by a higher incidence of *E. coli* in blood and a higher incidence of systemic lesions (pericardium and liver) in the higher EST × early feeding compared to the higher EST × delayed feeding treatment groups. Additionally, higher severity of total colibacillosis lesions (tMLS) and worse growth performance in higher EST × early feeding compared to higher EST × delayed feeding was found. This can likely be explained by the higher incidence of systemic *E. coli* infection, because system infection impairs heart and liver function and demands energy to fight the infection that cannot be used for body growth. Wijnen et al. (2022) also found some indications that EST can negatively impact the effect of early feeding. In that study, early fed broilers incubated at a lower EST of 36.7°C during late incubation tended to show higher 1st week mortality compared to early fed broilers incubated at control EST. It was speculated that this interaction may have been caused by higher incidence of yolk sac infection due to the combination of worse navel condition in lower EST incubated chicks and an expected higher bacterial load after early feeding. This proposed mode of action cannot explain the interaction between a higher EST during mid incubation and early feeding that was found in the current study. In the current study, no difference in navel condition was found between EST treatment groups and 1st week mortality was low and comparable in all treatment groups (average 0.6%). Only minor differences in chick quality at hatch between EST groups were found in the current study. For instance, YFBM and RY did not differ between EST groups and this is in consistency with previous findings (Wijnen et al., 2020a). Consequently, the effect does not seem to be directly related to chick quality characteristics at hatch and it can only be speculated what biological mechanism caused the interaction that was found in the current study.

Systemic colibacillosis develops when *E. coli* passes through the respiratory tract to the bloodstream and overwhelms the systemic defense (Matthijs et al., 2005). Possibly both a higher EST during mid incubation and early feeding lowered post hatch systemic immune responses. Early feeding may cause long term immunomodulation via for example alterations in gut microbiota (Brisbin et al., 2008; den Hartog et al., 2016), oral tolerance (Klipper et al.,

2001), or fatty-acid metabolism (Cherian, 2015). Studies have shown indications that early fed broilers show lower inflammatory responses than delayed fed broilers (Juul-Madsen et al., 2004; Gonzalez et al., 2011; Ao et al., 2012; Simon et al., 2015). This has not been shown for a higher EST during mid incubation, but higher incubation temperatures from mid incubation onwards resulted in worse developed lymphoid organs at hatch (Oznurlu et al., 2010; Liu et al., 2013; Flores et al., 2016; Leandro et al., 2017). Furthermore, a higher EST applied only during mid incubation altered peripheral blood lymphocyte composition as well as jejunum and bursa morphology at hatch (Wijnen et al., 2020b). These alterations also might have long-term effects, resulting in lower post hatch systemic immune responses. The fact that in the current study a higher EST during mid incubation resulted in a lower survival probability compared to a control EST suggests that a higher EST during mid incubation negatively affected embryo development, although that was not reflected in the chick quality characteristics at hatch, and that post-hatch immunocompetence was impaired. Early feeding could have degraded inflammatory immune responses that were already relatively low after a higher EST during mid incubation, which synergistically resulted in an inadequate response to a systemic *E. coli* infection.

Regardless of EST, early fed broilers showed lower local *E. coli* infections compared to delayed fed broilers, expressed by a lower incidence of local lesions. Probably this caused the lower overall colibacillosis lesion score (tMLS) and the tendency for a higher percentage of broilers with no lesions in early compared to delayed fed broilers. Furthermore, early feeding resulted in lower LNvar of standardized body weight deviations during the 13 days p.i. compared to delayed feeding. This indicates that in early compared to delayed fed broilers the negative deviations in body weight due to colibacillosis were either less severe or that recovery was faster or a combination between both (Berghof et al., 2019c; Poppe et al., 2020; van der Zande et al., 2020). The difference in resilience to local *E. coli* infection between feeding strategies may also be the result of a difference in inflammatory response. The inflammatory response seems to play a larger role in the susceptibility to colibacillosis compared to the adaptive immune response as differences in susceptibility are for instance not related to maternal antibodies (Ask et al., 2006b; Ariaans et al., 2008; Dwars et al., 2009; Peng et al., 2018; Alber et al., 2021). As indicated in the previous paragraph, early fed broilers may show lower inflammatory responses compared to delayed fed broilers. Severity of air sac lesions can be explained by inflammatory responses, especially excessive infiltration of macrophages (Dwars et al., 2009; Matthijs et al., 2009), so lower inflammatory responses in early fed broilers than in delayed fed broilers may explain the lower local *E. coli* infection that was found in the current study. In general, the immune system of early fed boilers seems to be ahead in development compared to delayed fed broilers, especially during the first weeks of life (Dibner et al., 1998; Shira et al., 2005; Panda et al., 2010; Hollemans et al., 2020). At first sight, a head start of approximately 48 hours in development might seem limited. However, the broiler immune system is immature at the moment of hatch and it develops rapidly during the first week of

age (Mast and Goddeeris, 1999; Shira et al., 2003). The first exogenous feed intake after hatch further stimulates this rapid development (Dibner et al., 1998) and consequently, early fed broilers showed higher resilience to local *E. coli* infections compared to delayed fed broilers.

In conclusion, this study was the first to show that EST during mid incubation and post hatch feeding strategy interact on broiler resilience to colibacillosis induced at day 8 of age. A higher EST of 38.9°C during mid incubation in combination with early feeding resulted in worse systemic resilience, whereas at a constant EST of 37.8°C feeding strategies did not differ in resilience to systemic infection. Regardless of EST during mid incubation, early fed broilers had higher resilience to local *E. coli* infections compared to delayed fed conspecifics. Consequently, early feeding could be considered as a strategy to enhance broiler disease resilience to infectious diseases as long as eggs are incubated at the current standard of a constant 37.8°C EST.

ACKNOWLEDGMENTS

The authors wish to thank Lagerwey hatchery for providing the eggs and incubators. We would also like to thank animal caretakers and other team members from the animal research facility. We are grateful for the assistance of Tom Berghof, Akke Kok, and Lisette Graat for their help with statistics on survival analysis or BW deviations, HatchTech colleagues (Jeroen Snijders and Thijme Heijink), WUR colleagues (Bjorge Laurenssen, Thijs Almekinders, Stephanie Melis), and especially Wageningen University students Serge Alindekon and Iris Jansen.

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CHAPTER 7

GENERAL DISCUSSION

INTRODUCTION

Despite a wide range of preventive measures, broilers will unavoidably encounter pathogens at some point in their lifespan. Infection cannot always be prevented. Particularly at risk are young broilers that still have an immature immune system or immunocompromised broilers. Once a pathogen has infected the broiler, it can induce disease and cause inflammation, pain, lesions, or even death. Furthermore, body growth will be retarded, resulting in lower profit. These negative scenarios can be prevented if the broiler is able to resist infection or if the broiler is able to cope with the pathogen with minimal clinical symptoms. This phenomenon is called 'resilience'. Resilience can be described as the ability to cope with disturbances and reorganize with minimal loss of function (Folke et al., 2010; Colditz and Hine, 2016). A resilient broiler has a lower chance of becoming infected and, once infected, it will show milder disease symptoms and a faster recovery than a broiler with a lower resilience.

It has been shown in many animal species that early life experiences, often referred to as 'perinatal conditions' (conditions surrounding birth), can affect later life immunocompetence (Rutherford et al., 2012). Perinatal conditions may therefore alter later life resilience. One perinatal condition of broilers that may potentially affect later life disease resilience is the post-hatch feeding strategy. In the poultry industry, chicks either have direct access to feed and water after hatch (defined as '**early feeding**') or, for practical reasons, access to feed and water is delayed for up to 72 hours (defined as '**delayed feeding**'). Studies have shown that these feeding strategies in early life can alter later life immune responses to antigens (Shira et al., 2005; Simon et al., 2015; Lamot et al., 2016), but whether early and delayed fed broilers differ in disease resilience remains unknown. Another perinatal condition that may affect broiler resilience in later life is incubation temperature. Incubation temperature has a major effect on embryo development and chick characteristics at hatch (Romanoff, 1936; Decuypere and Michels, 1992; Lourens et al., 2005), but its effects on broiler resilience, and how it interacts with post-hatch feeding strategy, is unknown.

The aim of this thesis was to study whether post-hatch feeding strategies, different eggshell temperature (**EST**) patterns during incubation, and the interaction between both, affect broiler resilience. Previous studies have withheld broilers from feed and water for 12, 24, 48, 60, and 72 hours. In this thesis delayed fed broilers were withheld for 48 hours because it was shown by de Jong et al. (2017) that this duration can already have long term effects on broiler growth performance. Besides, it is believed to be close to the duration that is found in commercial practice. We hypothesized in the General Introduction that early feeding would result in higher broiler resilience compared to 48 hours delayed feeding. We also hypothesized that a higher EST of 38.9°C during the second week of incubation in combination with a lower EST of 36.7°C during the last week of incubation or from day 17 of incubation onwards (referred to as 'late incubation') would optimize chick quality at hatch and post-hatch broiler resilience compared to a constant EST of 37.8°C. Furthermore, we hypothesized that optimal

chick quality at hatch would enhance the beneficial effects of early feeding on broiler disease resilience.

The origin of these hypotheses is described in the General Introduction (Chapter 1). The hypotheses were studied through three experiments that are presented in Chapter 2 – 6. This chapter will integrate the findings from these experiments to discuss whether the hypotheses can be verified or not. First it will be discussed how resilience can be interpreted in the current thesis, regardless of treatment effects. Subsequently, the effects of perinatal conditions on broiler resilience are discussed, followed by the effects of alternative incubation temperature patterns on embryo development and chick characteristics at hatch, before closing with main conclusions, practical implications, and suggestions for future research.

INTERPRETATION OF RESILIENCE

As previously described, the level of resilience is indicated by the 'loss of function'. Functional loss can be expressed with various biomarkers. In this thesis, functional loss was studied by measuring retardation of body growth, morbidity (e.g. lesions), and mortality during necrotic enteritis or during colibacillosis. Necrotic enteritis was induced by inoculation of *Eimeria spp*. at day 21 of age and repeated *C. perfringens* inoculation during the 5 days thereafter (Chapter 5). Colibacillosis was induced by intratracheal inoculation of avian pathogenic *E. coli* at day 8 of age (Chapter 6). Three aspects of resilience, which together comprise the total functional loss, were investigated: resistance, tolerance, and recovery (Schneider and Ayres, 2008; Bishop, 2012).

Resistance

A resilient broiler is assumed to have higher resistance, which is defined as the capability to withstand an infection. In the General Introduction of this thesis it was suggested that this could be studied by measuring the timespan between exposure to a pathogen until the first loss of function (Figure 1A). Chapter 5 indicates that this timespan could be studied successfully by measuring retardation of body growth during necrotic enteritis. Once retardation in body growth was expressed as average daily gain (**ADG**), a clear pattern of functional loss occurred in which the timespan between inoculation of *Eimeria* and onset of first loss of ADG could be measured (Figure 1B). However, Chapter 6 shows that resistance cannot always be studied by measuring retardation of body growth. Colibacillosis did cause retardation of body growth, but retardation occurred immediately (within 1 day) after inoculation with *E. coli* (Figure 1C). Consequently, resistance to colibacillosis did not occur or could at least not be determined through body weight retardation in this experiment because of the short timespan between exposure to the pathogen and the first effects on body growth.

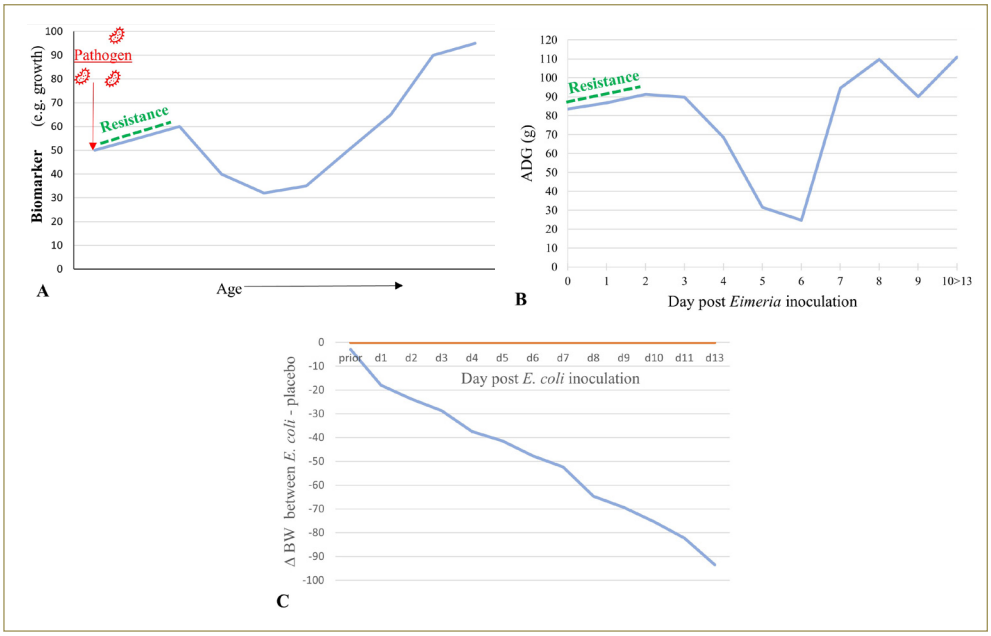

Figure 1. Graphical representation of resistance **A**) theoretical pattern **B**) functional loss in ADG during necrotic enteritis **C**) BW of *E. coli* inoculated broilers relative to placebo broilers.

Some broilers do not show any functional loss. These broilers were probably able to resist infection, for instance, because pathogens could not pass through epithelial barriers or mucus layers. This could be considered as the ultimate form of resistance. It can be studied by measuring the percentage of broilers that do not show any functional loss, such as organ lesions, after inoculation with the pathogen (referred to as 'incidence'). Disease incidence can be studied by measuring the percentage of animals with organ lesions (referred to as 'morbidity'). However, there is one concern when studying disease incidence through morbidity. To determine morbidity, broilers are dissected at specific time points post inoculation. At these time points, a broiler may have resisted infection and, in such circumstances, morbidity data will represent disease incidence; however, it is also possible that the pathogen is still present in the broiler's body and that it may cause infection later, or that the broiler already recovered at the moment of dissection. It can be speculated that this ambiguity can be solved by very regular dissections of broilers during the disease period. However, despite the fact that this raises ethical concerns and is practically impossible in some experimental settings, it may not even solve the problem either. For example, in the last experiment (Chapter 6) dissections were performed at 3 hours, 12 hours, 1 day, 2 days, 4 days, and 7 days post inoculation of *E. coli*. Amongst others, incidence of systemic infection was determined during these dissection moments by measuring whether *E. coli* bacteria were present in the blood. Results showed that the highest incidence occurred at 12 hours post inoculation and, moreover, that the incidence at this dissection moment differed significantly from the other dissection moments ($P < 0.01$;

Figure 2). Theoretically, the observed incidence of systemic infection could have been higher if broilers would have been dissected at an additional dissection moment, for instance at 8 hours post inoculation. It would then be possible that a higher incidence of systemic infection is found for treatment 'blue' in Figure 2 at 12 hours post inoculation, whereas actually systemic infection was higher for treatment 'green', but this occurred at 8 hours post inoculation when no dissections were performed.

The total incidence (= sum of incidence of separate dissection moments) may therefore provide the best representation of the actual incidence, with the footnote that dissection should have been performed frequently enough. How often is 'frequent enough' depends on the disease challenge model, the predictability of the disease responses, and the rapidity of fluctuations in disease responses. Therefore conclusions about incidence of disease that are based on morbidity should be drawn with some restraint and they should be combined with other data concerning functional loss to draw sound conclusions about disease resilience.

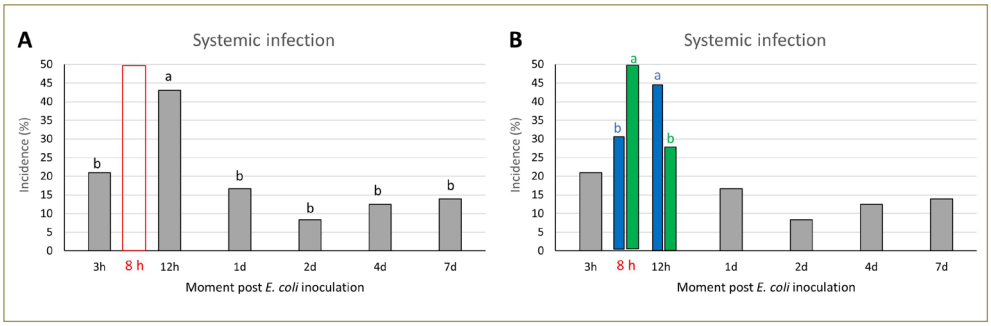

Figure 2. Incidence of system infection (bacteria in blood) at 6 moments post inoculation with *E. coli*. Grey bars indicate real data.
A. The red bar indicates theoretical data if broilers would have been dissected at an additional moment at 8 h post inoculation.
B. Blue bars and green bars indicate theoretical data from two different treatments.

Tolerance

A resilient broiler is assumed to have higher tolerance, which is defined as the capability to keep functional loss to a minimum once infected. Tolerance can be studied by measuring to what extent functional loss occurs (Figure 3A). During necrotic enteritis, tolerance could be studied by measuring the severity of loss in ADG (Figure 3B), whereas this was impossible during colibacillosis, because the difference in BW between diseased and placebo broilers was gradually increasing over time (Figure 3C). In this experiment, the placebo group enabled us to calculate deviations of body weight from broilers with colibacillosis compared to healthy broilers, which are an indicator of resilience (Berghof et al., 2019a; Poppe et al., 2020; van der Zande et al., 2020). In these analyses, standardized body weight deviations from the total observation period are combined to calculate the skewness and variance of these deviations.

Skewness of these deviations captures the severity of body weight deviations and represents the tolerance aspect of resilience. The variance of deviations captures both the severity and duration of deviations and so represents both the tolerance and recovery aspects of resilience, without disentangling them (Berghof et al., 2019b).

Tolerance can also be studied by measuring the extent to which morbidity occurs. However, as discussed in the previous paragraph, the number of dissection moments after inoculation may affect the morbidity outcome and may even provide a distorted picture. Specifically if kinetics in disease response fluctuate very rapidly. For example, broilers were dissected at 6, 7, and 14 days post *Eimeria* inoculation and severity of intestinal lesions differed significantly between 6 and 7 days post inoculation (Chapter 5). It remains unknown if the extent of lesion severity would have been worse if broilers were dissection at additional moments before or after 6 / 7 days post inoculation and, consequently, care should be taken when drawing conclusions based on morbidity data. Similarly to total disease incidence as an indicator of resistance, total disease severity (= sum of severity of morbidity at separate dissection moments) may provide the best representation of the actual severity and, therefore, could be used to study the tolerance aspect of resilience.

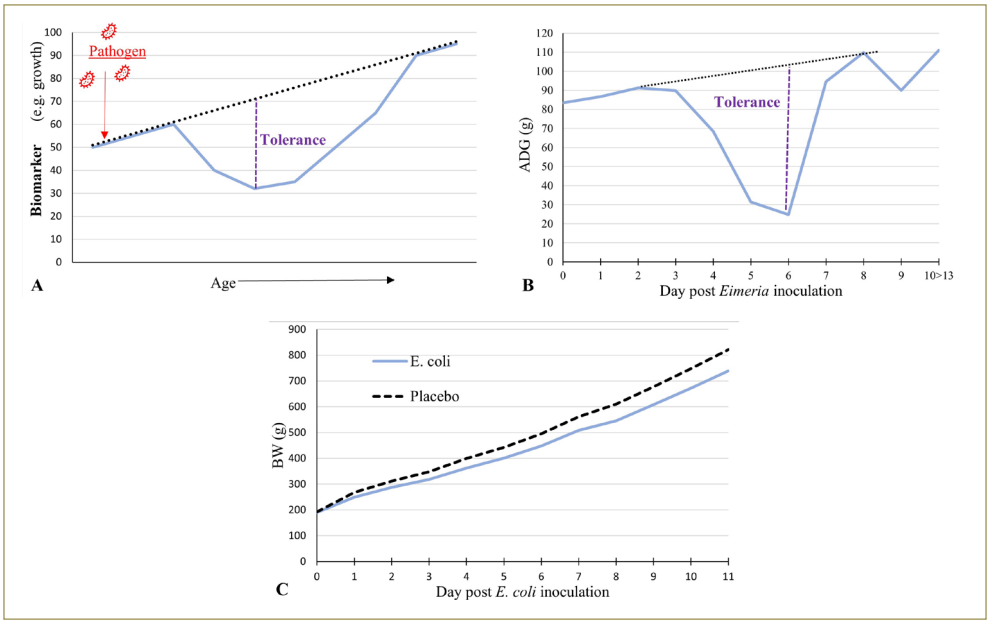

Figure 3. Graphical representation of tolerance **A**) theoretical pattern **B**) ADG during necrotic enteritis **C**) BW during colibacillosis.

Recovery

A resilient broiler is assumed to recover faster from an infection. Recovery was defined as the capability to return to a normal state after infection and it can be studied by measuring the total duration of functional loss (Figure 4A). In the given example, the broiler is capable of returning to a state that is similar to a broiler that was not affected by infection. This represents optimal recovery. It is also possible that a broiler recovers from an infection and that it returns to a homeostatic state during the remaining life, but that it is not capable to return to a similar level as unaffected broilers (Figure 4B).

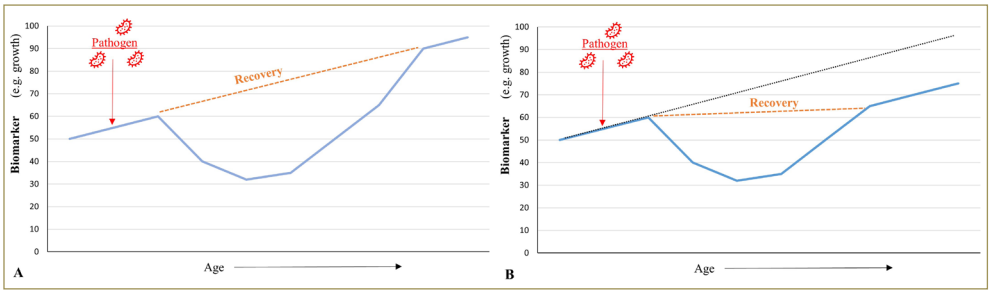

Figure 4. Graphical representation of recovery **A**) theoretical pattern full recovery **B**) theoretical pattern recovery to new homeostatic state.

Recovery is the return of the biological function to its norm; for example, when ADG returns to its pre-infection rate. However, during necrotic enteritis, recovery seemed to occur very rapidly, as was expressed in the recovery of ADG within 1 day from 10 g to 90 g (Figure 1B). In contrast, during colibacillosis, diseased broilers did not show recovery in body weight withing the period of daily weighing the birds (Figure 1C). The rapid recovery of ADG within 1 day during necrotic enteritis and the late or non-recovery during colibacillosis made it difficult to measure recovery speed by studying retardation in body growth. As indicated in the previous paragraph, the variance of body weight deviations during colibacillosis does include the recovery aspect, and it may provide more detailed information on the broilers that did not recover during the observed time period. However, variance of body weight deviations cannot be disentangled from the tolerance aspect that is also included in this parameter. Alternatively, lag-one autocorrelation of body weight deviations can be calculated. Lag-one autocorrelation captures the duration of functional loss, so it represents the recovery aspect of resilience (Berghof et al., 2019b). Lag -one autocorrelation can only be interpreted as recovery under the condition that recovery occurred within the period that body weights were measured. Body weights of all ~ 1800 broilers were measured individually to enable lag-one autocorrelation calculations during 13 days post *E. coli* inoculation, but, unfortunately, this effort was in vain as recovery did not occur within this period (Figure 1C).

Recovery can also be studied by measuring the disappearance of morbidity, although findings indicate that this may not be a very good indicator of recovery in the current thesis. For instance, colibacillosis lesions were scored in organs of 72 broilers at 5 weeks post inoculation. In 32 % of these broilers, lesions were still present. Blood and bacterial swabs from the air sacs of these broilers were also investigated for the presence of *E. coli,* and it was subsequently found that in the vast majority of these broilers (> 60%), live *E. coli* were no longer present (Table 1). This indicates that lesions or remnants thereof may persist despite that broilers already had recovered from the infection, compromising the value of morbidity data as an indicator for recovery.

Table 1. Presence of *E. coli* in blood or in air sacs at 5 weeks post inoculation with these bacteria in broilers with organ lesions.

Lesions	E. *coli* in blood	E. *coli* in air sacs	No E. *coli*
Air sacs only	0 % (0 / 13)	23 % (3 / 13)	77 % (10 / 13)
Systemic (air sacs / pericardium / liver)	10 % (1 / 10)	30 % (3 / 10)	60 % (6 / 10)
No lesions	0 % (0 / 49)	6 % (3 / 49)	94 % (46 / 49)

Mortality is probably the most reliable factor to study the recovery aspect of resilience as it represents the most definite form of 'non-recovery'. Mortality can be seen as the ultimate functional loss. It should be noted that mortality can affect the outcome of all previously indicated aspects. For example, broilers that died cannot be included in the incidence of morbidity data. Necropsy can be performed, but the moment of death likely differs from post-mortem analysis of broilers that were selected for dissection. Furthermore, broilers that were dying often gradually lost body weight several days prior to death, and thus showed a different pattern in retardation of body growth compared to broilers that were recovering.

Resilience in this thesis

To summarize, all three aspects of resilience were measured in this thesis by various biomarkers, depending on the type of disease and limitations in the number of dissection and/or weighing moments. **Resistance** can be interpreted in two ways; 1) the timespan between exposure to a pathogen until first functional loss occurs, and 2) disease incidence (= percentage of broilers that do not show any functional loss at all). In the current thesis, resistance was studied by measuring the timespan between *Eimeria* inoculation and first loss of ADG during necrotic enteritis and by measuring the total incidence of morbidity during colibacillosis (Table 2). **Tolerance** was studied by measuring the severity of ADG loss during necrotic enteritis and the variance and skewness of body weight deviations during colibacillosis, as well as the total severity of morbidity during colibacillosis. **Recovery** was studied by measuring mortality rates. In the remainder of this general discussion, conclusions about broiler resilience will therefore be drawn based on these biomarkers (Table 2).

Table 2. Overview of biomarkers that could be measured to study the three complementary aspects of resilience for each disease model in the current thesis.

Broiler resilience			
Disease model	Resistance	Tolerance	Recovery
Necrotic enteritis	ADG	ADG	Mortality
Colibacillosis	Total morbidity incidence	- Total morbidity severity - Skewness of BW deviations	Mortality
		Variance of BW deviations	

PERINATAL CONDITIONS AND BROILER RESILIENCE

Post-hatch feeding strategy

Post-hatch feeding strategy often affected broiler resilience independent of incubation temperature. These findings will now be discussed in the current paragraph. In some cases, the effect of post-hatch feeding strategy depended on incubation temperature. These cases will be discussed in the section 'Incubation temperature × post-hatch feeding strategy'.

Resilience

Several indications were found which suggest that early fed broilers have higher resilience compared to delayed fed broilers. All three aspects of resilience seem to be affected. Firstly, a higher disease resistance was found in early fed broilers, which was expressed by a lower total incidence of lesions in air sacs as well as a tendency for a lower total incidence of colibacillosis (Chapter 6). Secondly, disease tolerance was higher in early fed broilers compared to delayed fed broilers, as shown by lower variance of body weight deviations during colibacillosis in early compared to delayed fed broilers. Thirdly, early feeding appears also to affect the capability to recover from a disease, since mortality during the 2 weeks post onset of necrotic enteritis tended ($P = 0.06$) to be lower in early compared to delayed fed broilers (Chapter 5).

Other studies that investigated the effects of post-hatch feeding strategy on broiler health did not study broiler resilience through all three complementary aspects of resilience; resistance, tolerance, recovery. Besides, in some of those studies 'early feeding' consisted of a hydrated supplement instead of feed and water, or first access was provided at placement on the farm instead of directly after hatch. Nevertheless, findings from these studies are in line with findings from the current thesis. For example, broilers that had access to a hydrated nutritional supplement directly after hatch showed higher growth when challenged with a high dose of coccidiosis vaccine at day 14 of age compared to 48 hours delayed fed broilers (Dibner et al., 1998). Broilers that had access to a glutamine supplemented diet directly after hatch showed lower mortality and less severe lesions after oral inoculation with *Eimeria* oocysts

at day 22 of age compared to 48 hours delayed fed broilers (Yi et al., 2005). Furthermore, during necrotic enteritis at 3 weeks of age, early fed broilers had higher relative growth rate compared to 48 hours delayed fed broilers. Lower counts of *C. perfringens* were found in the ileum if the early fed diet contained mannooligosaccharides (Ao et al., 2012). Early fed laying hens showed higher body weight gain and feed intake after an *Eimeria* challenge day 53 of age as well as after an infectious bronchitis challenge at day 92 of age compared to delayed fed hens, but this was confounded with different incubation temperatures and rearing conditions (Walstra et al., 2010). Early fed broilers compared to 72 hours delayed fed broilers showed higher feed intake and lower sickness behaviour when challenged with a combination of *E. coli* lipopolysaccharide with human serum albumin at day 28 of age (Simon et al., 2015).

Taking together the findings from these studies and findings from the current thesis, there is a growing body of evidence that early feeding supports broiler resilience to infectious diseases in later life. Several biological mechanisms have been proposed through which post-hatch feeding strategy may affect broiler resilience in later life (reviewed by Zubair and Leeson, 1996; Noy and Sklan, 1997; Friedman et al., 2003; Noy and Uni, 2010, Willemsen et al., 2010; de Jong et al., 2017). Some examples are through the uptake of residual yolk (**RY**), through nutrient availability for physical development, and through development of the immune system with possible long-term modulations to the type of immune responses. These are discussed in the following sections.

Residual yolk

It has been suggested that early feeding stimulates the uptake of RY by activating digestive enzymes and/or increasing peristaltic movements (Noy and Sklan, 1998; Sklan and Noy, 2000). Although some studies showed that early feeding resulted in lower weight of the RY until approximately 4 days of age compared to delayed feeding (Romanoff, 1944; Bierer and Eleazer, 1965; Moran and Reinhart, 1980; Noy and Sklan, 1998; Noy and Sklan, 2001; El-Husseiny et al., 2008; Bhanja et al., 2009; Cardeal et al., 2021), weight of the RY did not differ at 48 hours and 96 hours of age between post- hatch feeding strategies in the current thesis (Chapter 4) and in other studies (Bigot et al., 2003; Gonzales et al., 2003; Franco et al., 2006; Gaglo-Disse et al., 2010; Tabedian et al., 2010; van den Brand et al., 2010; Hollemans et al., 2020a). Effect of post-hatch feeding strategy on weight of the RY are therefore inconsistent and it can be questioned if a higher total uptake of RY explains the higher broiler resilience that is found in early compared to delayed fed broilers.

Perhaps early fed broilers utilize the RY content differently compared to delayed fed broilers, without affecting the weight of the RY. For instance, it has been found that early fed chicks mainly utilized amino acids and minerals from the RY, whereas delayed fed chicks utilized lipids and moisture (Romanoff et al., 1944; Moran and Reinhart et al., 1980; Hopcroft et al., 2020). It can be speculated that chicks can also actively use the RY for development of the immune system, as the RY has been suggested to play a role in immune development

(Murakami et al., 1992; Osama and Huwaida, 2013). Yolk is rich in IgY that are deposited in the yolk by the hen (Kowalczyk et al., 1985; Ulmer-Franco et al., 2012). The humoral immune response of a neonatal chick is fully dependent on this maternal IgY, as their own immunoglobulin production of IgM and IgA isotypes is still immature in early life (Lawrence et al., 1981). If early feeding stimulates specific uptake of IgY, this may enhance disease resilience in early fed broilers.

Immune organ development

Another biological mechanism that may explain the difference in disease resilience between post-hatch feeding strategies concerns the availability of nutrients. Some egg nutrients, such as carbohydrates, vitamins, and specific fatty acids, are depleted in the RY at hatch moment (Yair and Uni, 2011; Cherian, 2015). Some of these nutrients, such as omega-3, are essential nutrients, which cannot be synthesized by the chick itself. If these essential nutrients are not provided with feed directly after hatch, there will be a period of shortage during early life. Many organs, including immune related ones, develop rapidly during early life, and their optimal development demands adequate nutrient and energy resources (Kwak et al., 1999; Rudrappa and Humphrey, 2007). Early feeding can provide these nutrients, whereas delayed feeding extends the period of nutrient shortages post hatch and thus limits immune organ development. It has previously been shown by others that delayed feeding can result in lower weight of the bursa or spleen relative to body mass (Dibner et al., 1998; Simon et al., 2014). Relative weights of the bursa at 48 hours or 96 hours after hatch were not affected by post-hatch feeding strategy in the current thesis (Chapter 4), although it could be the case that some crucial alterations in immune organ development are not reflected in the weight of the organ. Furthermore, only the bursa was investigated in the current thesis and perhaps other organs that are involved in the immune system were affected as well. Intestinal weights were lower and morphology was worse at 48 hours and 96 hours in delayed compared to early fed broilers (Chapter 4) and the gut can be considered to some extent to be an immune organ as well. After all, the gut mucosa and the epithelial barrier are the first line of defense against enteric pathogens and immune response to enteric pathogens can modulate the general immune system of a chicken (Friedman et al., 1994; Broom, 2019). If lower development of immune organs cannot be compensated at later ages, this may explain the lower broiler resilience that was found in delayed fed broilers.

Maturation of the immune system

The higher resilience in early compared to delayed fed broilers may be explained by an accelerated maturation of the immune system. Biliary IgA, systemic IgM natural antibodies, mature B-cells (expressed by BU-1 marker), germinal centers in cecal tonsils, and colonization of the hindgut and bursa with lymphocytes were all found at younger ages in early fed broilers

compared to delayed fed broilers (Dibner et al., 1998; Juul-Madsen et al., 2004; Shira et al., 2005; Simon et al., 2014).

Maternal IgY levels in newborn chicks decrease gradually over time during the first 2 weeks of age (Gharaibeh and Mahmoud, 2013). In the current thesis, lower IgY levels were often found in early fed compared to delayed fed broilers (Chapter 4). Additional data shows that 48 hours delayed fed broilers have approximately the same IgY levels as early fed broilers 48 hours later (Figure 5). This could be an indication that early feeding indeed accelerates maturation of the immune system, but that it may just be a head start in maturation and not an improvement of development.

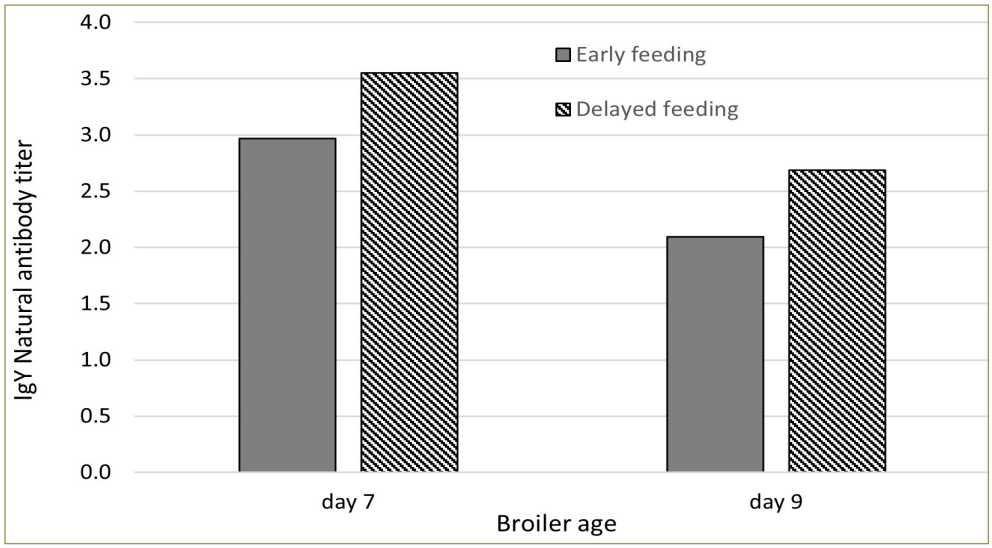

Figure 5. IgY natural antibodies against keyhole limpet hemocyanin titer at day 7 and 9 of age in broilers after early feeding compared to delayed feeding.

It can be speculated that a head start in development of the immune system will provide short term support of broiler resilience for early fed chickens up until the adaptive immune system of delayed fed broilers is functional, which seems to occur during the first 2 weeks of age (Friedman et al., 2003; Shira et al., 2005; Taha-Abdelaziz et al., 2018). In that case, effects of post-hatch feeding strategy on broiler resilience will probably mainly be pronounced at young ages and to lesser extent in older broilers (> 2 weeks old). This may explain why differences in broiler resilience were more evident in the response to colibacillosis, which was induced at day 8 of age, compared to necrotic enteritis that was induced at day 21 of age. Hollemans (2020) concluded in his thesis that early feeding may improve resistance to infectious diseases during the first week of age because higher IgM natural antibodies were found in early compared to delay fed broilers up to 7 days of age, whereas at older ages no difference in systemic humoral immunity was found.

It should be noted though that broiler age was not the only difference between the experiments that were conducted within this thesis. The age of the parent flock (Cardeal et al., 2021) and the type of disease (respiratory vs intestinal) may also explain why differences in resilience were more pronounced during the colibacillosis experiment. Nevertheless, mortality during necrotic enteritis at 3 weeks of age tended to be lower in early compared to delayed fed broilers ($P = 0.06$, $\Delta = 6.6\%$). Furthermore, others studies showed beneficial effects of early feeding on disease response up to at least 28 days post-hatch, as was previously described in more detail (paragraph '3.1 Resilience'). Possible explanations for such long-term alterations due to early feeding include immunomodulation through alterations in gut microbiota (Brisbin et al., 2008; Kabir, 2009; den Hartog et al., 2016), oral tolerance (Klipper et al., 2001), dietary compounds (reviewed by Cherian, 2015), gene expression (Xu et al., 2012; Powell et al. 2016), or trained innate immunity (Netea et al., 2020), but some of these concepts have been introduced only relatively recently and need further investigation for their contribution to broiler resilience.

Stress

Stress through post-hatch feeding strategy may contribute to alterations in broiler resilience. Feed restriction is known to cause stress in chickens aged 3 weeks or older, expressed by higher plasma corticosterone concentrations, increased peripheral heterophil to lymphocyte ratio, and/or behavioural changes (Buckland et al., 1974; Nir et al., 1975; Freeman et al., 1980; Scott et al., 1983; Hocking et al., 1996; Najafi et al., 2015; Pál et al., 2015). It has often been speculated that, in contrast to older birds, feed restriction is less stressful in neonatal birds because the latter possess RY in their abdominal cavity that can be transported directly into the gastrointestinal tract for up to 5 days post hatch (Esteban et al., 1991; Jeurissen et al., 1991; Noy et al., 1996; van der Wagt et al., 2020). However, the current thesis indicates that the availability of residual yolk probably does not prevent stress in chicks that have delayed access to feed and water after hatch. In Chapter 4, 2.5 x higher corticosterone level was found in 48 h-delayed fed broiler at 48 hours after hatch compared to early fed broilers and this is in line with previous findings (van de Ven et al., 2013). Corticosterone increases the number of peripheral heterophils and decreases the number of lymphocytes (Shapiro and Schechtman, 1949; Weller and Schechtman, 1949; Bannister, 1951; Glick, 1958; Wolford and Ringer, 1962; Bishop et al., 1968) and consequently a short-term impairment of disease resilience may occur in delayed fed broilers.

Stress is not always disadvantageous for immunocompetence. It has been shown that acute stress (2 h) enhanced leukocyte responses in the skin (Dhabhar, 2002). The author suggested that acute stress prepares the immune system for an infection by leukocyte redistribution. This indicates that although corticosterone affects blood lymphocyte profiles, acute stress may not results in immunosuppression or impaired disease resilience. Oppositely, chronic stress decreases the amount of Natural Killer cells and increases susceptibility to infectious diseases

(reviewed by Dhabhar, 2014). Whether delayed feeding causes chronic stress may depend on the duration. Findings from Khosravinia and Manafi (2016) suggest that feed restriction of 36 hours may not yet be stressful to the neonatal chick, because chicks showed no attempts to escape from a non-fed area, whereas a duration of 48 hours resulted in a significant increase of jumping attempts. Therefore, it can be speculated that short durations of delayed feed access after hatch (< 48 h) may not have a negative impact on the immune system, whereas longer durations (> 48 h) may be a risk factor for chronic stress and immunosuppression and, consequently disease resilience may be impaired.

It has been suggested that stress during early life can alter fear responses of chickens in later life (Janckzak et al., 2006), possibly through gene expression in the hypothalamus (Xu et al., 2012). Hedlund and Jensen (2019) showed that laying hens that experienced more stress during hatchery processes had higher increases in corticosterone levels during manual restraint at 6 day of age and more physical injuries at 140 days of age. This suggests that stress during early life of chickens may impair the ability to cope with stress during later life. Therefore it can be speculated that stress by delayed feeding impairs the ability of a broiler to cope with stress during later life and possibly this has an additional decreasing effect on resilience to infectious diseases as was discussed in the previous paragraph. No evidence was found for a long term increased stress response through delayed feeding during early life, but further research is recommended, as this was not the scope of the current thesis and was therefore not thoroughly investigated with just one behavioural test at day 13 of age (Chapter 4).

Incubation temperature × post-hatch feeding strategy

Early feeding does not always guarantee a higher broiler resilience compared to delayed feeding. In some cases the effect of post-hatch feeding strategy was dependant on incubation temperature. These interactions will be discussed now. It should be noted that regardless of post-hatch feeding strategy, incubation at temperatures other than a constant EST of 37.8°C lowered broiler resilience (discussed later in paragraph '3.3 Incubation temperature'). Therefore, any EST pattern other than a constant EST of 37.8°C is referred to as 'suboptimal EST'.

Suboptimal EST × post-hatch feeding strategy: Resilience

Findings from the current thesis indicate that early fed broilers that are incubated at suboptimal EST may have more difficulties to cope with systemic, but not local, infections compared to delayed fed broilers that are incubated at suboptimal EST. At suboptimal EST, early fed broilers had a lower resistance to systemic infection with pathogenic *E. coli* compared to delayed fed broilers, which was expressed by 11 % higher incidence of *E. coli* in blood and 10 % higher incidence of systemic lesions (pericardium + liver) (Chapter 6). Furthermore, at suboptimal EST, early fed broilers had a higher severity of colibacillosis lesions compared

to delayed fed broilers, which suggests that the tolerance to a systemic *E. coli* infection was also worse in the suboptimal EST × early feeding group compared to the suboptimal EST × delayed feeding group.

Resilience to necrotic enteritis did not show an interaction between incubation temperature and post-hatch feeding strategy (Chapter 5). In this experiment a remarkably high 1st week mortality occurred in all treatment groups (average 7.7 %). First week mortality tended ($P = 0.07$) to be higher in the suboptimal EST × early feeding group compared to the suboptimal EST × delayed feeding group in this experiment (+ 5.5 %) (Chapter 4). Lamot et al. (2016) also found remarkably high mortality in their study (approx. 8.6% between day 0 – 28), and again mortality was especially high in early compared to delayed fed broilers. Possibly, incubation temperature was suboptimal in their study too, but EST was not reported. De Jong et al. (2017) found in their meta-review of 75 studies on early feeding that 48 hours of delayed feeding resulted in higher mortality during rearing (day 0 – 42) than early feeding. This suggest that, in general early feeding has a positive effect on survival, but some factors exist which cause early feeding to be detrimental to the chick. Findings from the present studies suggest that suboptimal incubation temperature may be one such factor.

Suboptimal EST × post-hatch feeding strategy: immunomodulation

The combination of a suboptimal EST pattern with early feeding may have suppressed the immune responses of broilers to such an extent that responses were too low to overcome systemic infections. It has been shown that a suboptimal incubation temperature can suppress the post hatch immune system through suboptimal development of lymphoid organs (Oznurlu et al., 2010; Leandro et al., 2013; Liu et al., 2013) or through gene expression of cytokines that are involved in inflammatory immune responses (Saleh and Al-Zghoul, 2019). A suboptimal EST altered morphology of bursal follicles at hatch moment and the blood lymphocyte profile from hatch up to at least 3 days of age in the current thesis (Chapter 3 and 4). These alterations in immune related parameters may be evidence of a weaker immune systems, although this requires further investigation. As previously described, post-hatch feeding strategy can also cause persistent modulations in the type of immune response (reviewed by Taha-Abdelaziz et al., 2018). Indications have been found that early feeding suppresses inflammatory immune responses compared to delayed fed broilers, such as lower expression of MHC-II on B-cells, altered fatty acid metabolism, higher vitamin-E status, higher cyclooxygenase expression, lower cytokine production, and higher biliary IgA (Juul-Madsen et al., 2004; Gonzalez et al., 2011; Ao et al., 2012; Simon et al., 2015; Hollemans et al., 2020b; Proszkowiec-Weglarz et al., 2020). It has been shown in humans that immunosuppression can be beneficial during infections with *Streptococci* or *E. coli* (Husson et al., 2016), which could explain the higher broiler resilience after early feeding discussed earlier. However, it has also been shown that the immune system can be suppressed to such an extent that phagocytosis of intestinal pathogens like *Salmonella* is too low (Sijben et al., 2001; Cherian et al., 2007). Thus, early feeding as

well as suboptimal EST may synergistically suppress immune responses, which could explain the lower disease resilience to systemic infection in this treatment group compared to the suboptimal EST × delayed feeding group. EST and post-hatch feeding strategy interacted on natural IgY antibody response during necrotic enteritis (Chapter 5) and natural- and *E.coli* IgY and IgM antibody response during colibacillosis (unpublished). Antibody titers were inconsistently lower, higher, or did not differ at all in this thesis, so whether this indicates immunosuppression cannot be concluded with certainty. Additionally, only antibodies were measured in the current thesis, whereas cellular immune responses probably played a large role in resilience to systemic infections (French et al., 2020).

EST pattern × delayed feeding

The higher EST × delayed feeding group and the control EST × delayed feeding group did not differ in incidence of colibacillosis or systemic lesions, nor did growth performance after disease either. Furthermore, regardless of post-hatch feeding strategy, a higher EST of 38.9°C during the second week of incubation lowered the probability to survive colibacillosis compared to a constant EST of 37.8°C. Mortality is assumed to be a strong indicator of lower resilience, as it indicates non-recovery or 'ultimate loss of function'. Therefore, although lower severity of colibacillosis lesions and lower incidence of *E. coli* in blood was found in higher EST × delayed feeding compared to control EST × delayed feeding (Chapter 6), no strong evidence was found that delayed fed broilers had a higher resilience if they were incubated at a higher EST of 38.9°C during the second week of incubation compared to a constant EST of 37.8°C.

Incubation temperature

Broiler resilience was also affected by a main effects of incubation temperature independent to post-hatch feeding strategy. It was hypothesized that a higher EST of 38.9°C during the second week of incubation in combination with a lower EST of 36.7°C during late incubation would result in higher broiler resilience compared to a constant EST of 37.8°C, but this hypothesis could not be accepted.

Resilience

Neither a higher EST of 38.9°C during the second week of incubation, nor a lower EST of 36.7°C during late incubation, nor the combination thereof, improved broiler resilience compared to a constant EST of 37.8°C. In fact, resilience seemed to be deteriorate due to these alternative EST patterns. A higher EST during mid incubation appeared to lower the ability to recover from a disease, expressed by a lower probability to survive colibacillosis compared to a constant EST of 37.8°C (Chapter 6). A lower EST during late incubation seems to impair all three aspects of resilience compared to a constant EST of 37.8°C; a lower disease resistance was expressed by a shorter timespan between *Eimeria* inoculation and first loss in ADG; a

lower disease tolerance was expressed by a higher loss in ADG during necrotic enteritis and a higher number of oocysts in feces of broilers that were incubated at a lower EST during late incubation (Chapter 5); and a lower recovery ability was expressed by a higher 1st week mortality (Chapter 4) and a tendency for higher overall mortality during rearing (Chapter 3). Jabbar et al. (2020) also showed that a lower EST during later incubation increased mortality rates during rearing. Thus, despite our hypothesis that the alternative EST patterns would improve broiler resilience compared to a constant EST of 37.8°C, the opposite was found. The effects of alternative EST patterns on embryo development and chick quality characteristics at hatch will be discussed in the following paragraphs and may explain the lower broiler resilience in later life.

Immune system

A higher EST of 38.9°C during the second week of incubation and a lower EST of 36.7°C during late incubation may have impaired development of immune organs compared to a constant EST of 37.8°C. At hatch moment, the density of cells within bursal follicles seemed to be affected in both directions by these alternative EST patterns (Chapter 3). These findings indicate that bursal development was affected to some extent by the alternative EST patterns, and it can be speculated that the alterations expresses impaired development. Other studies, with high incubator air temperatures, confirm that physical development of lymphoid organs during embryogenesis and morphology at hatch moment can be impaired by incubation temperature (Oznurlu et al., 2010; Liu et al., 2013; Sozcu and Ipek, 2015; Flores et al., 2016; Maatjens et al., 2016a; Leandro et al., 2017). It is not known whether alterations in immune organ development that are found at hatch persist during rearing, but perhaps impaired physical development of the bursa may have had a lasting effect on the broiler's immunocompetence, explaining the worse resilience to necrotic enteritis and colibacillosis after a higher EST of 38.9°C during the second week of incubation or a lower EST of 36.7°C during late incubation compared to a constant EST of 37.8°C. For example, the alternative EST patterns affected the ratio of lymphocytes to heterophils from hatch up to at least 3 days of age (Chapters 3 and 4). Furthermore, the alternative EST patterns resulted in a higher Newcastle disease titer at slaughter age compared to a constant EST of 37.8°C (Chapter 3). This indicates that the alternative EST patterns had long lasting effects on the broiler's adaptive immune response. However, EST had no effect on other parts of the humoral immune system like natural antibodies against keyhole-limpet hemocyanin (**KLH**) (Chapters 3 and 5). These natural antibodies against KLH seem to be correlated with disease resilience in laying hens (Star et al., 2007; Sun et al., 2011; Wondmeneh et al., 2015; Berghof et al., 2019c), but layer-type chickens differ significantly in their immune response compared to broilers (Koenen et al., 2002; Simon et al., 2016). To conclude, a higher EST of 38.9°C during the second week of incubation and a lower EST of 36.7°C during late incubation affected bursal follicle

morphology at hatch moment. This may have affected immune responses in later life to such an extent that resilience was impaired, but this needs further investigation.

Organ development

Besides immune organs, incubation temperature may have altered physical development of other organs that can affect broiler resilience. For example, a lower EST of 36.7°C during late incubation seems to affect villi and crypt morphology in the jejunum from hatch up to 2 days of age (Chapter 3 and 4). Early intestinal development seems to affect immune responses in later life (reviewed by Lilburn and Loeffler, 2015), therefore suboptimal development of the gastrointestinal tract during embryogenesis may indirectly result in suboptimal development of immunocompetence during later life. Furthermore, a lower EST of 36.7°C during late incubation resulted in a higher weight of the heart relative to yolk-free body mass (**YFBM**) (Chapter 2 and 4). Maatjens (2016) showed similar results regarding the heart weight and suggested that a higher relative heart weight at hatch could be predictive for improved later life performance. The current thesis however, does not concur, since broilers that were incubated at a lower EST of 36.7°C during late incubation had higher relative heart weight at hatch but growth performance upon slaughter age was lower (Chapter 2). Additionally, it was shown that broilers that were incubated at an even lower EST of 35.6°C during late incubation had the highest heart weights at hatch but the lowest body weight at day 7 post-hatch (Maatjens et al., 2016a+b). Similar results were seen in late hatching chicks that had larger yolk utilization and higher relative organ weights, including the heart, but had depressed growth performance during rearing (van de Ven et al., 2011). Furthermore, we showed that higher relative heart weights that were found at hatch occur only very short term as they disappeared between 48 and 96 hours after hatch (Chapter 4). Moreover, as previously indicated, broiler resilience was impaired by lowering EST to 36.7°C during late incubation. Heart weight at hatch does not seem to predict later life performance or resilience of broilers.

Predictability of resilience by chick quality at hatch

Chick quality characteristics at hatch that were measured in this thesis may not be greatly predictive of disease resilience in later life. Some chick quality characteristics, such as YFBM or RY, were not different between EST treatments, whilst disease resilience was reliably impaired under alternative EST patterns. Other chick quality characteristics did differ between EST treatments, but the results were reversed between the alternative EST patterns and despite this, both alternative EST patterns resulted in worse disease resilience. For instance, a higher EST of 38.9°C during the second week of incubation resulted in a shorter incubation duration, longer chick length, lower blood glucose level, lower relative heart weight, lower cell density within bursal follicles, and a lower blood heterophil: lymphocyte ratio compared to constant EST of 37.8°C, whereas all these characteristics showed a significant opposite effect when

lowering EST during late incubation (Chapter 2 and 3). Therefore, none of these chick quality characteristics seem to be predictive of later life resilience to pathogenic infections.

INCUBATION TEMPERATURE AND EMBRYO DEVELOPMENT

In the General Introduction of this thesis it was hypothesized that a higher EST of 38.9°C during the second week of incubation and/or a lower EST of 36.7°C during late incubation would enhance embryo development in terms of YFBM and organ development compared to a constant EST of 37.8°C. Subsequently, optimal embryo development through these alternative EST patterns was expected to result in a higher broiler resilience. However, as previously discussed, the opposite occurred and broiler resilience was negatively affected by these alternative EST patterns. In this section we discuss how the alternative EST patterns may have affected actual embryo development differently to what was hypothesized at initiation of this thesis.

Higher EST mid incubation

A higher EST of 38.9°C during the second week of incubation increases embryo body temperature as embryos act poikilothermic for the major part of the incubation period (Dietz and van Kampen, 1994; French et al., 1997). As a result of increased embryo body temperatures, metabolism and embryo development may be accelerated, which results in a higher heat production (Pearson et al., 1991; Lourens et al., 2011). This was indicated by a higher heat production and a higher YFBM of embryos at embryonic day (**E**) 14 and E16 when EST was raised to 38.9°C from E7 onwards (Lourens et al., 2007; Nangsuay et al., 2016; Nangsuay et al., 2017). In this thesis, embryo development and heat production were not investigated. Therefore, it cannot be said with certainty that YFBM during the second week of incubation was affected in the current thesis as well. Instead, chick characteristics were measured at hatch, where at no difference in YFBM was found between a 38.9°C EST during the second week of incubation compared to a constant EST of 37.8°C (Chapter 2 & Chapter 6). This indicates that either the rate of embryo development was not affected during the second week of incubation at a higher EST or that any increase in YFBM that may have been created in the second week of incubation decreased during the last week of incubation.

Comparable studies do not provide a decisive answer. On the one hand, van den Brand et al. (2021) found no difference in embryonic heat production during the second week of incubation between eggs from 37 – 45 week old broiler parent flocks that were incubated at 38.9°C or 37.8°C EST during 6 batches, which suggests that the rate of embryo development was not affected. On the other hand, during an additional experiment which has not been published yet, eggs were incubated at either a constant EST of 37.8°C or a 38.9°C EST during the second week of incubation (and 37.8°C EST during wk1 and 3) with YFBM begin

determined every 2 days from E8 onwards and at E19 and hatch. The higher EST during the second week of incubation resulted in a higher YFBM at E14, E16, and E18 compared to a constant EST of 37.8°C, whereas this difference disappeared at E19 and at hatch (Figure 7). It should be noted that during incubation YFBM was determined at chronological age, whilst at hatch YFBM is determined at biological age and chicks that are incubated at higher EST during mid incubation hatch several hours earlier than those incubated at a constant EST of 37.8°C. Taken together, although a higher EST during mid incubation can increase embryo developmental rate during mid incubation, this is not consistently seen, and differences in development may diminish during the last week of incubation anyway.

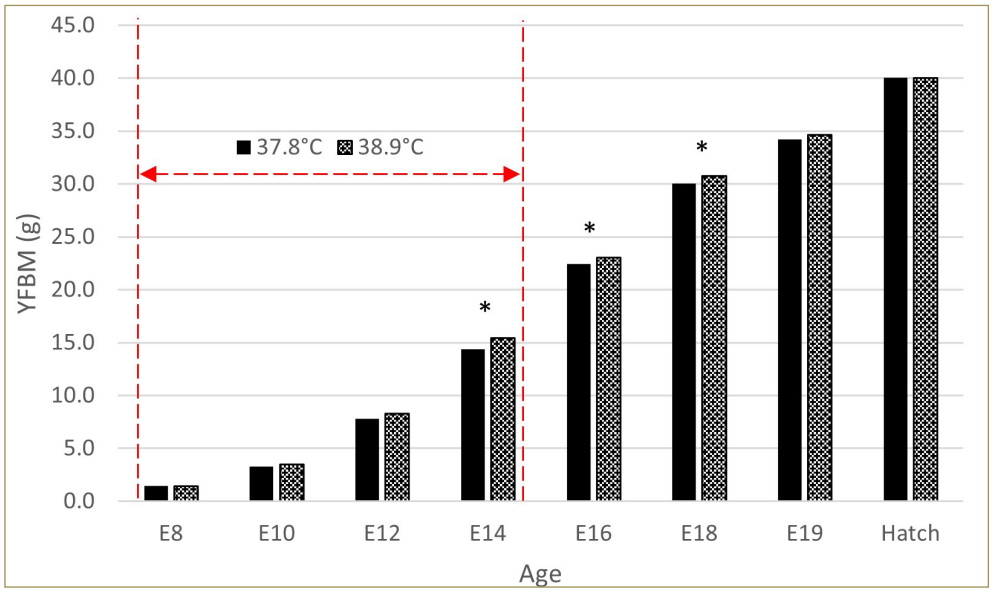

Figure 7. Yolk-free body mass (**YFBM**) at different embryo ages (**E**) and at hatch when incubated either at constant 37.8°C or 38.9°C eggshell temperature during the 2nd wk of incubation (indicated with red dashed lines (EST wk 1 +3 = 37.8°C). * Indicates significant difference between treatments (P<0.05). (data from Priester et al., unpublished)

Lower EST late incubation

We hypothesized that lowering EST during the last week of incubation to 36.7°C would lower the metabolic rate and thereby the oxygen demand and synergistically interact with a higher EST during mid incubation on embryo development and chick quality at hatch. Chapter 2 shows that this hypothesis was incorrect, because EST week 2 and week 3 did not show an interaction on any chick quality characteristics at hatch and post hatch growth performance (Chapter 2 and 3).

A lower EST during late incubation did not affect the conversion of yolk into body mass during embryo development to the extent that was first expected. This expectation was based

on findings from Maatjens et al. (2016b) who showed that a lower EST of 36.7°C from E15, E17, or E19 onwards, resulted in higher YFBM and relative weight of the liver and heart at hatch (Maatjens et al., 2016a). However, a higher YFBM at hatch was not consistently found after lowering EST to 36.7°C during late incubation in our experiments or in those of others (Maatjens et al., 2014a; Almeida et al., 2016; Morita et al., 2016; van den Brand et al., 2019; Chapter 2 and 4). RY weight at hatch moment was also not different between a constant EST of 37.8°C and a lower EST during late incubation in this thesis and in previous studies (Maatjens et al., 2014a; Almeida et al., 2016; Maatjens et al., 2016a; Morita et al., 2016; Hamidu et al., 2018; Chapter 2 and 4), which indicates that the amount of yolk utilized seems not to be affected by a lower EST during late incubation.

One very consistent observation is that of a higher relative heart weight at hatch when lowering EST during late incubation (Maatjens et al., 2014a; Maatjens et al., 2016a; van den Brand et al., 2019; Chapter 2 and 4). One theory proposes that a lower EST during late incubation positively supports glycogen synthesis and this possibly lowers the need for catabolism of proteins from the heart (Maatjens, 2016). However, this theory is not consistently supported by other indicators of protein metabolism like plasma lactate, uric acid, or blood pH and bicarbonate (Maatjens et al., 2014b; Maatjens et al., 2017; van den Brand et al., 2019). Further refuting this theory is that it has also been shown that hearts that had higher weights, due to hyperoxia during incubation, did not differ in protein content compared to smaller hearts (van Golde et al., 1998), which suggest that weight of the heart may not be correlated to protein content of the heart. Another theory proposes that the higher glucose availability for lower EST incubated embryos increased utilization of yolk and albumen protein for heart development (Maatjens, 2016). However, whilst the heart specifically seems to be consistently affected, the same is not true for results on YFBM. Strong evidence for these glucose-driven mechanisms in which either protein is removed from the heart (through catabolism) under suboptimal temperatures, or added to the heart (through anabolism) under optimal temperatures, is lacking.

Instead, we here present a new theory to explain the higher relative heart weight that is consistently found when lowering EST to 36.7°C during late incubation compared to a constant EST of 37.8°C. Although chick embryos act poikilotherm and full thermoregulatory response develops only after hatching (Dietz and van Kampen, 1994 ; French et al., 1997), embryos have been observed to temporarily increase their heat production in response to cooling of the egg (Romijn and Lonkhorst, 1955; Tazawa et al., 1989; Lourens et al., 2006; Szdzuy et al., 2008). The increase in heat production was often relatively low and only temporary, but Szdzuy et al. (2008) suggested that embryos do intent to respond to colder temperatures by increasing their heat production, but that only a mild increase in heat production is observed because the embryo is limited by oxygen availability. Other homeothermic mechanisms to cold, such as vasoconstriction, may also occur. It has previously been shown that vasoconstriction can indeed occur in avian embryos, as evidenced by a decrease in chorioallantoic blood flow in

embryos from laying hens when incubator air temperature was lowered to 31.5°C or 34.5°C during E12 to hatch (Nichelman and Tzschentke, 1999). During vasoconstriction, blood pressure increases and high blood pressure may eventually lead to a higher heart weight (Petersen et al., 2017). This suggests that the higher relative heart weight at hatch after lower EST during late incubation may not be the result of enhanced embryo development but from a thermogenic response. Therefore, the increased heart weight under lower EST does not seem to occur due to enhanced development of the heart.

Homeothermic responses may result in less efficient conversion of yolk into embryo body mass because these responses require energy. It has been shown that 37.4°C EST during the last week of incubation compared to a constant EST of 37.8°C resulted in lower absolute measure of lipids, moisture, dry matter, and crude protein in RY at hatch moment whereas weight of the RY and weight and composition of the YFBM did not differ between incubation temperatures (Almeida et al., 2016). Thus, yolk was utilized less efficiently at lower EST during late incubation, perhaps because energy was used for homeothermic responses instead of body development. The lower yolk conversion efficiency suggests that lowering EST during late incubation may negatively affect embryo development, which could explain the indications of worse chick quality characteristics at hatch that were found in the current study, such as the minor but significant and consistent decrease in chick length (Chapter 2 and 4), the shorter jejunum villi length and crypt depth, and the higher cell density in bursal follicles when EST was lowered to 36.7°C from E14 onwards (Chapter 2). In line with this, lower EST incubated day-old chicks may have a later onset of thermoregulatory ability after hatch compared to control EST, which was indicated by a tendency for a higher preferred ambient temperature (Δ = 1.4°C; Chapter 2). This further supports that lower EST during late incubation may negatively affect chick development, because post-hatch thermoregulatory ability can be used to classify maturity (Whittow and Tazawa, 1991).

To conclude, a higher EST of 38.9°C during the second week, a lower EST of 36.7°C during late incubation, and the interaction between them did not optimize embryo development and chick characteristics at hatch in terms of YFBM. Instead, these alternative EST patterns probably impaired embryo development as they negatively affected broiler resilience. The first study on EST patterns already showed that a constant EST of 37.8°C during incubation seems to result in most optimal embryo development and chick quality at hatch (Lourens et al., 2005). Since then, no sound evidence has been generated that any other EST pattern will positively affect embryo development and performance at later age and this thesis did not either. Therefore, a constant EST of 37.8°C is still considered to be optimal.

MAIN CONCLUSIONS

- Perinatal conditions can affect broiler resilience to respiratory and intestinal infectious diseases in later life.

- Direct access to feed and water after hatch results in higher resilience to infectious diseases in broilers compared to 48 hours delayed access.

- At a suboptimal eggshell temperature pattern, direct access to feed and water after hatch lowers resilience to systemic, but not local, infections compared to 48 hours delayed access.

- A constant eggshell temperature of 37.8°C results in higher broiler resilience to infectious diseases compared to a higher eggshell temperature of 38.9°C during the second week and/or a lower eggshell temperature of 36.7°C during late incubation.

PRACTICAL IMPLICATIONS

- Relatively small deviations in eggshell temperature (1.1°C) can already have a considerable effect on broiler resilience during rearing. This emphasizes the importance of incubation for the entire production chain and it shows the need for a low variation in temperature within incubators.

- Early feeding could be used as a strategy to increase broiler resilience to infectious diseases in later life, lowering the need for antibiotics. It should be noted though that resilience is not a holy grail. Infections may still occur, but the impact may be lower in early compared to delayed fed broilers. Preventive measures such as sanitary protocols and biosecurity are still needed.

- Early fed broilers seem to prefer a lower ambient temperature compared to delayed fed broilers up to at least 12 days of age, probably because feeding increases their development and heat production. This indicates that there is an increased risk of overheating early fed broilers. This should be considered during storage, transportation, and rearing.

- Eggshell temperature during the last days of incubation can have a major impact on broiler resilience, and it can interact negatively with early feeding. This should be considered when incubating eggs in different types of hatching systems such as conventional hatchers, hatchery fed, or on-farm hatching.

FUTURE RESEARCH OPPORTUNITIES

- This thesis found that disease resilience was affected by perinatal conditions. Some biological mechanism were proposed to explain these results and these mechanisms could be investigated using a fundamental approach in future studies.

- Effects of post-hatch feeding strategy can be influenced by incubation temperature. This should be taken into account when reviewing literature or when conducting future studies on effects of post-hatch feeding strategy.

- Small deviations in eggshell temperature seem to have a large impact on broiler resilience during rearing. This emphasized that care should be taken when comparing literature on incubator air temperature to eggshell temperature. It is proposed to monitor and report eggshell temperature in future studies as an accurate reflection of the actual incubation temperature.

- Perinatal conditions have a significant effect on resilience of fast-growing broilers. Other poultry species such as layer-type chicken or turkey, have comparable management conditions during their perinatal phase. Furthermore, most poultry diseases are pathogenic to all poultry species. This suggests that it may be worthwhile to investigate the effects of perinatal conditions in other chicken breeds and poultry species as well.

- This thesis showed that very frequent observations of biological functioning is vital to study all three aspects of resilience. Ideally, biological functioning is monitored continuously in future studies on animal resilience, for instance with data collected through precision livestock farming techniques (e.g. sensor data).

- This thesis showed that delayed feeding may cause stress to the neonatal chick. Stress during early life can have long term effects. Long term effects on stress mechanisms were not thoroughly investigated in the current thesis so further research is suggested.

- Early fed broilers seem to be ahead in development in terms of body growth, immune responses, and thermoregulatory capacity compared to delayed fed broilers, specifically during the first weeks of age. Current management conditions, such as vaccination schedules, housing temperatures, or diet formulations, are often based on studies with conventional delayed fed broilers. Possibly some management conditions have to be reformulated for early fed broilers.

- Chick quality characteristics at hatch do not always seem to be good predictors for later life. Consequently, to study the effects of incubation conditions on chick development it is advised to include the post-hatch period.

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SUMMARY

The increasing global concern about rising antimicrobial resistance led to a growing interest in alternative approached to prevent and control infectious diseases. One approach is to support animal resilience. Resilience can be defined as the ability of an animal to cope with environmental disturbances and reorganize with minimal loss of function. Resilience to infectious diseases can be studied by measuring three complementary aspects; **resistance** to becoming infected, **tolerance** during infection, and **recovery** from the infection.

Optimal early life conditions can have long-lasting effects and may therefore support animal resilience. Until now, in broilers, effects of early life conditions on resilience against infectious diseases were largely unknown. The moment of first access to feed and water after hatch was hypothesized to affect broiler resilience in later life. In practice, broiler chicks either have access to feed and water directly after hatch (defined as '**early feeding**') or access to feed and water is delayed for up to 72 hours (defined as '**delayed feeding**'). Secondly, incubation temperature may affect broiler resilience in later life as it can also affect growth performance in later life. A constant eggshell temperature (**EST**) of 37.8°C (**Control EST)** was considered optimal. However, studies found indications that a higher EST of 38.9°C during the second week of incubation (**Higher mid EST)** as well as a lower EST of 36.7°C during late incubation (**Lower late EST)** may enhance embryo and chick development. Furthermore, the interaction between a higher mid EST with lower late EST (**alternative EST pattern**) was not investigated yet. We hypothesized that the alternative EST pattern would synergistically interact with early feeding to enhance broiler resilience in later life.

Experiment 1 investigated the effects of alternative EST patterns on chick characteristics at hatch, especially immune organ development, and later life immune responses and growth performance. Results in this study did not show an interaction between higher mid and lower late EST, nor a main effect for most chick characteristics at hatch and growth performance. Lower late EST resulted in higher relative weight of various organs, such as the heart, compared to control EST, but other chick characteristics did not differ. Lower late EST resulted in a lower growth performance untill slaughter age. This was probably the result of a delayed hatch moment and consequently a shorter time to grow, rather than impaired growth performance **(Chapter 2).** Furthermore, no interaction between higher mid and lower late EST was found for most measurements on immune organ development, blood lymphocyte profiles, and immune responses during rearing. Higher mid EST resulted in lower cell density within bursal follicles and a lower blood heterophil: lymphocyte ratio compared to control EST, and this was reversed for lower late EST. Natural antibodies during rearing were not affected by EST. Newcastle disease titer at slaughter age was higher for higher mid EST compared to control EST. Furthermore, a tendency for higher mortality during the total rearing period was found for a lower late EST compared to a control EST. So, indications were found that

incubation temperature could affect broiler's later life and their immune system, but effects on resilience remained unknown yet **(Chapter 3)**.

Because no interaction was shown between higher mid and lower late EST, the effects of these were tested separately in the following two experiments, and post-hatch feeding strategy (early or delayed by 48 hours) was included**.**

Experiment 2 investigated late incubation temperature, post-hatch feeding strategy, and the interaction between them on physiological regulatory systems during early life and resilience to necrotic enteritis during later life. There was no interaction of EST x feeding strategy, except that the lower late EST × early feeding group tended to result in higher 1st week mortality compared to the other 3 treatment groups. Early feeding resulted in higher body development at 2 days and 4 days post hatch. Additionally, early feeding reduced stress on the short term, expressed by lower plasma corticosterone, but no later life effects on stress response were found. Early feeding stimulated thermoregulatory ability to at least 12 days post hatch compared to delayed feeding, expressed by lower preferred ambient temperatures **(Chapter 4)**. Necrotic enteritis was induced at day 21 of age. Mortality and body weights were measured daily for 2 weeks and disease morbidity was assessed at day 28 and 29. No interaction between EST and post-hatch feeding strategy on disease resilience was found. A lower late EST lowered resilience to necrotic enteritis compared to control EST, expressed by higher losses in ADG and higher oocysts in feces. Early feeding tended to enhance resilience to necrotic enteritis compared to delayed feeding, expressed by a tendency for lower mortality rate. Disease morbidity, expressed by intestinal lesions at day 28 and 29, was not affected by EST nor post-hatch feeding strategy **(Chapter 5)**.

Experiment 3 investigated the effects of mid-incubation temperature, post-hatch feeding strategy, and the interaction between them on broiler resilience to colibacillosis. Colibacillosis was induced at 8 days of age. Body weights were measured daily for 13 days, morbidity was evaluated at 6 moments post *E. coli* inoculation, and mortality was noted daily. Results showed that higher mid EST with early feeding resulted in a higher incidence of systemic infections, expressed by a higher percentage of broilers with *E. coli* in blood and lesions in the liver and/or pericardium, whereas the amount of *E. coli* and severity of systemic lesions were not affected. At control EST, systemic infection was not affected by feeding strategy and severity of total mean lesions score was lower for early compared to delayed feeding. Early feeding resulted in a lower incidence of local infection, lower daily body weight losses, and higher growth performance until slaughter A higher mid EST lowered the probability to survive colibacillosis.

To conclude, early feeding results in higher resilience to infectious diseases in broilers compared to delayed feeding. At suboptimal EST, early feeding lowers broiler resilience to systemic, but not local, infections compared to delayed feeding. Both higher mid EST and lower late EST result in a lower resilience to infectious diseases in broilers compared to a control EST (37.8°C).

ACKNOWLEDGEMENTS

I started this PhD adventure because I wanted to learn every single fact within a certain field of animal sciences. Now, five years later, I know that this was an impossible goal and that this goal is an illusion rather than an achievable skill. "*Scientist learn to use the word 'impossible' with greatest caution*" - (Wernher von Braun). Although I did not achieve my initial goal, I did learn an awesome lot about incubation and poultry in general. Above all, I learned that gaining knowledge was relatively irrelevant compared to the opportunity to meet many new inspiring people. The collaborations during projects and experiments, the social talks during coffee breaks, the yearly team outings, and the drinks and diners during conferences or other events were probably the most valuable part and they brought me everlasting memories. I want to thank anyone of you for that! Furthermore, I would also like to express my sincere gratitude to some specific persons as follows.

Ten eerste wil ik **Henry van den Brand** en **Bas Kemp** bedanken voor het vertrouwen in mij als PhD kandidaat voor HatchTech. Jullie hebben tijdens mijn promotieonderzoek een aantal keer een essentiële rol gespeeld. Uiteraard bij de start van het traject, maar ook halverwege toen ik op het punt stond om een carrière stap binnen HatchTech te maken. Daarnaast hadden jullie ieder altijd scherpe en waardevolle input op onze artikelen. Bas, tijdens onze gesprekken wist jij me altijd op het juiste moment en op een fijne manier een spiegel voor te houden, zodat ik eigenlijk niets anders meer kon dan het me je eens zijn. Doorgaans resulteerde dit in het schrappen van een stuk tekst (zelfs met glimlach!) en dat is het geheel zeker ten goede gekomen. Henry, bedankt dat de deur bij jou altijd open heeft gestaan voor een gezellig praatje of de gekste vragen. Ik denk dat er wereldwijd maar weinig mensen zijn die net zoveel kennis over broeden paraat hebben als jij. Je hebt me overigens altijd de indruk gegeven dat gekke vragen niet bestaan. Je open houding, geduld en respect naar de ander kon ik altijd erg waarderen. Daarnaast deed je ooit een uitspraak over zelfvertrouwen van PhD studenten die ik erg pakkend vond en die me geholpen heeft tijdens de rest van het traject.

Ten tweede een speciaal woord van dank aan alle **(ex) OBP-ers**; Bjorge Laurenssen, Joop Arts, Monique Ooms, Ilona van den Anker-Hensen, Marcel Heetkamp, Manon van Marwijk, Mike Nieuwland, Ger de Vries-Reilingh, Rudi Koopmanschap, Kevin Lin en Fleur Bartels. Sommigen van jullie waren al bij ADP toen ik in jullie team terecht kwam. Ik heb me altijd direct welkom gevoeld. Bovendien hebben jullie me echt gesteund tijdens de ziekte van mijn moeder. Ik kon ook altijd bij jullie terecht voor praktische vragen en doordat jullie me overal bij betrokken kreeg ik een kijkje in de keuken van een grote diversiteit aan projecten. Dit heeft er uiteindelijk sterk aan bijgedragen dat ik het aandurfde om op een PhD vacature te solliciteren. Dank daarvoor! Ook bedankt voor jullie flexibiliteit en hulp op de gekste

tijdstippen gedurende mijn experimenten en aansluitend in het lab. Mochten jullie ooit in de buurt van Chicago aan de Maas zijn, schroom dan niet om een bak koffie te komen drinken!

Bjorge ik verwacht dat jij de 3 uurkes vurraf voortaan ook wel in je eentje kan hendelen, want er zijn geloof ik weinig zaken die jij niet kan hendelen. Misschien kom je ooit nog tot de wijsheid dat eieren en kippen veel fascinerender zijn dan de anus van een varken, rottende baarmoeders, berenzaad of weet ik veel wat waar je me allemaal ooit nog meer in hebt weten te betrekken. ADP heeft een hele goede kracht aan jou waar ze zuinig op kunnen zijn! Enorm bedankt voor je harde werk tijdens mijn experimenten, je flexibiliteit en je praktische oplossingen die je vaak precies op het juiste moment wist aan te dragen. Dank ook dat je mijn paranimf bent. Het is een blijk van waardering voor het voorgaande, maar ook een blijk van waardering voor jou als fijn persoon.

Dan wil ik graag **Nanette van Hapert** en **Lora van der Kleijn** bedanken! Het is ontzettend fijn dat jullie elk verzoek snel en kordaat afhandelen. Als PhD-er kun je zeker op jullie expertise vertrouwen en hoef je je niet druk te maken dat er een deadline of belangrijk document vergeten zal worden. Dat is het zakelijke gedeelte, maar jullie vervullen daarnaast een hele belangrijke sociale rol binnen ADP. Althans, dat heb ik altijd zo ervaren, want het was erg gezellig om even binnen te lopen en met jullie te kletsen. Daarnaast wil ik jullie nog bedanken voor jullie gastvrijheid en (het aanbod van) een slaapplek. Dat ligt toch net even wat fijner dan een autostoel. Lora, ik hoop je in de toekomst ook nog zo nu en dan tegen het lijf te lopen op een feestje, maar dan graag zonder poncho en stortbuien.

Thanks to all the **(former) PhD candidates and colleagues from ADP**. It was a pleasure to collaborate with anyone of you during experiments, to drink coffee, go for a walk on the campus, and to watch chicken diarrhoea photo's on a 6 x 3 mtr screen during conferences. Special thanks to **Marieke Priester** as we started our PhD together at HatchTech. Unfortunately it turned out that it did not suit you, but I want to thank you for the nice start that we made together. I will not forget our trip to the ESPN in Barcelona (especially the roller coasters…). All the best with your awesome job at Avolare and I promise to visit it in the future. **Conny Maatjens**, we met each other during the final stage of your PhD and I still remember that you advised me to apply for a PhD job at HatchTech. You told me that the company and especially the collaboration with Inge en Carla would fit me very well. You were definitely right! Thank you for the great work that you have been doing on incubation temperature. I am probably the person that read your entire thesis the most often…. **Bahadir Can Güz**, it was a pleasure to collaborate with you during one of the experiments and it was an honour to be your paranymph. I wish you all the best in your future career and most of all in your private life. Hopefully we will meet again during a poultry conference, in Turkey, or during a visit to the Netherlands (you're always welcome!). **Jesse Heijmans**, thanks for the nice chats we had,

your helpfulness, the exchange in knowledge concerning feed and incubation and the drinks in Dubrovnik (I kindly request you to delete any photographs > 6 pm). I am sure we will meet again, but I want to use this opportunity to wish you every success in the last phase of your PhD trajectory and above all a lovely future with Hans and your family. **Maarten Hollemans**, thanks for having discussions with me about early feeding, for the coffee breaks, and for your hospitality and pizza. It was a pleasure to help with your experiment and perhaps we may collaborate in the future again. **Dr. van den Bosch**, dear Moniek, my deepest respect on how you managed to successfully complete your PhD! Your persistency, flexibility, and positivity probably helped you out a lot during the difficult phases and make you a very nice person to be around with. It was a pleasure to help during your huge experiment at Sterksel, although I won't miss the sleepy drives back home on Friday mornings. It was great to have someone with such a good sense of humor to chat with in the middle of the night during the last phase. This made me feel at comfort and helped me to relativize the stressful final stage. Thanks for everything and all the best with your future career and lovely family.

Marleen Verdaasdonk, Margriet Faber, Lara Olde Bolhaar, Lianne Cheung, Bart de Bont, Charlotte Visser, Maartje Frankena en Iris Jansen, jullie waren stuk voor stuk hele fijne studenten om mee samen te werken en jullie thesis begeleiden was zeker een van de allerleukste dingen uit mijn promotietraject! Dank voor jullie inzet, jullie kritische vragen en bovenal jullie gezelschap. Ik wens jullie al het goeds in een verdere carrière en hoop jullie nog eens tegen te komen in de pluimveesector of in de kroeg. **Serge Alindekon** thanks once again for your help during my experiment. It was a pleasure to have you as a student in our team, because you were always smiling, regardless of the approx. 20,000 broilers that we had to weigh or the precarious Dutch weather. If you ever need help during your PhD experiments please let me know (preferably in Benin).

Roos Molenaar, bedankt!
(Ik was eerst van plan om 3 A4-tjes vol te schrijven waarin ik mijn waardering uit wilde spreken over je co-promotorschap en dat je altijd positief bent en voor me klaar hebt gestaan en dat we qua schrijfstijl dan misschien niet op een lijn zaten, maar dat we desondanks toch met veel plezier hebben kunnen samenwerken en dat je me hebt geholpen om niet te verdwalen, maar ik heb dit niet gedaan omdat je me bovenal hebt geleerd om bij de essentie te blijven (al is dat met deze lange zin alsnog niet helemaal gelukt).

Eenieder van **Carus** bedankt voor de prettige samenwerking tijdens mijn experimenten en een extra blijk van waardering voor Sabine, Bert en Sherine. Jullie kippenexpertise, nauwkeurigheid en inzet zorgde ervoor dat ik camping Carus al vrij snel met een gerust hart durfde te verlaten.

Ik wil ook eenieder van broederij (en tevens camping) **Lagerwey** bedanken. Jullie waren altijd erg behulpzaam en flexibel wanneer ik jullie om een gunst kwam vragen. Ik ga altijd met veel plezier naar Lunteren en hoop dit nog vaak te mogen doen in de toekomst.

Alle zuiderburen van **Poulpharm** en bovenal Isaura Christiaens en Annelike Dedeurwaerder bedankt voor jullie hulp rondom het experiment! Mede door jullie is het zo goed verlopen en ik zal het dan ook aan eenieder aanraden om Poulpharm te overwegen. Dit experiment was zeker een van de hoogtepunten uit mijn promotietraject waar ik nog altijd met veel plezier op terugkijk. Ik hoop dat wij lompe directe Hollanders het niet te bont hebben gemaakt en dat we nog eens een pintje samen kunnen drinken.

De halve zuiderbuur **Mieke Matthijs** en haar collega **Andreas Papanikolaou, Thijs Almekinders** en **Stephanie Melis,** bedankt voor jullie flexibiliteit en grote inzet tijdens het laatste experiment en bedankt voor de fijne werksfeer waar jullie zo aan hebben bijgedragen! Ik sta bij jullie in het krijt. De afsluitende borrel van het experiment was wat studentikoos i.v.m. corona, dus ik stel voor dat we dit op 1 juli extra goed overdoen.

Vera Perricone, per favore usa Google Translate per tradurre quanto sopra in italiano, quindi sai che sono molto grato per il tuo aiuto! Qualsiasi persona che non parli italiano leggendo questo potrebbe ora pensare che parlo italiano e questo mi fa sembrare un po' più intelligente. Ma se si prendono la briga di metterlo anche in Google Translate, allora sono tristemente sbalordito.

Carla van der Pol en **Inge van Roovert-Reijrink**, bedankt voor jullie vertrouwen in mij. Ik kan me ons sollicitatiegesprek nog heel goed herinneren. Voor mijn gevoel klikte dat meteen en dat is ook wel gebleken tijdens de rest van mijn promotietraject. Jullie lieten me vrij wanneer dat kon en zaten me achter de vodden wanneer dat nodig was. No-nonsense, flexibiliteit en collegialiteit, scherpe input en een hele goede dosis humor (en jullie liefde voor Nederlandstalige muziek) heeft ervoor gezorgd dat ik dit promotietraject met veel plezier volbracht heb. Ik had me echt geen betere copromotoren kunnen wensen!

Ik wil ook alle andere **collega's van HatchTech** bedanken voor jullie bijdrage aan mijn promotietraject. Voor sommigen was die bijdrage erg duidelijk, zoals voor Thijme Hijink en Jeroen Snijders door hun hulp tijdens mijn experimenten. Voor anderen was die bijdragen misschien niet direct zichtbaar, maar ook jullie hebben me indirect geholpen door jullie oprechte interesse in mijn project of simpelweg door de altijd gezellige feestjes!

A special thanks to my **colleagues from Ukraïne**. I would like to express my deepest respect to anyone of you for your contribution to our company in this difficult situation. I have

never ever experienced such a courageous nation. You can be so proud on yourself and your families. Eventually love will conquer and your kids will be raised in a beautiful and peaceful country. Слава Україні!

Tjitze Meter, allereerst bedankt voor je investeringen in onderzoek in het algemeen. Ik ben ervan overtuigd dat dit echt wereldwijd invloed heeft op de progressiviteit van de pluimveesector. Heel inspirerend om te zien hoe jij onvermoeibaar met allerlei bedrijfsaspecten bezig kunt zijn en daarbij vaak een paar stappen vooruit denkt. Mijn respect heb je bovenal door je werknemers oprecht te waarderen, gelijk te behandelen waar mogelijk en door hen zoveel mogelijk te betrekken bij 'ons' mooie bedrijf. Ik kijk er naar uit om me hier nog meer voor in te zetten!

Lieve **schoonfamilie**; Lenette, bedankt dat je altijd oprechte interesse hebt getoond in mijn promotietraject, voor je attente appjes wanneer ik een belangrijk gesprek of presentatie had en dat je er altijd voor m'n meiden bent. Hans, bedankt voor de gezellige potten bier die we gedronken hebben en voor je keiharde werken waardoor we zoveel mooie uitstapjes en vakanties hebben mogen maken met zijn allen. Dennie, Anikie en Raf jullie bedankt voor alle gezelligheid tijdens deze uitstapjes!

Nala, ook bedankt. Ik weet nu waar de uitdrukking 'trouwe viervoeter' vandaan komt.

Lieve **zussen, broer** en **partners daarvan**, wat was het fijn dat ik op jullie kon terugvallen wanneer er werk aan de winkel was in eht weekend en ik wel wat hulp kon gebruiken. Mijn lokkertje van een kijkje in de keuken van hoogstaand wetenschappelijk onderzoek wierp zijn vruchten af en jullie stonden uiteindelijk met je volgescheten overal kiloknallers te wegen. Willem heb ik ditmaal maar ontzien in verband met zijn merkwaardige fobie, maar die had zijn portie ook al eens eerder gehad toen hij wekenlang niet heeft kunnen lopen na de beet van een schattig biggetje. Zonder dollen, mijn dank voor jullie hulp is groot! Daarnaast hebben jullie me de afgelopen tijd geholpen door een luisterend oor te zijn, door mij te ontlasten bij het regelen van allerlei familiezaken of door me mee op stap te nemen om de hele avond liedjes van Nederlands beste artiest te zingen. Ik weet bovendien dat ik altijd bij jullie terecht kan en dat alleen al is eigenlijk genoeg. Dank daarvoor!

Lieve **mam**, bij de start van dit promotietraject was het al vrij duidelijk dat je dit dankwoord niet zelf zou kunnen gaan lezen. Toch wil ik je nog even expliciet benoemen, dat verdien je! Onbewust heb je namelijk enorm bijgedragen aan deze stap in mijn leven. Je hebt me van kinds af aan tot je laatste snik perfect aangevoeld. Je gaf me zelfvertrouwen, je stimuleerde me om het onderste uit de kan te halen zonder daarbij een last op mijn schouders te leggen en bovenal heb je me geleerd om te genieten van het leven en daarbij iedereen in zijn waarde

te laten. Toen je ons verliet was ik dit even kwijt en heb ik je enorm gemist. Dat was dan ook meteen de enige keer in mijn leven dat je een negatief effect hebt gehad op mijn 'zakelijke prestaties', maar uiteindelijk gaf het me nou juist de motivatie om door te pakken tijdens moeilijke momenten. Deze titel is dus ook voor jou. Ik zal er eentje extra op je drinken mam!

Lieve **pap**, ons mam heeft dit natuurlijk niet alleen gedaan ook al wil je dit nog wel eens, bescheiden als je bent, proberen te beweren. Dat beweer je waarschijnlijk omdat je veel aan het werk was. Maar dit heeft er natuurlijk ook sterk aan bijgedragen dat ik überhaupt de kans heb gekregen om dit promotietraject te starten. Ik heb onbezorgd de opleidingen kunnen volgen die ik wilde en jij benadrukte daarbij altijd om mijn hart te volgen en te kiezen 'wat ik leuk vond'. Bedankt pap! Ik heb het overigens ook nooit zo ervaren dat je veel van huis was. Je was er altijd voor me en kwam voor me op wanneer dat nodig was. Je gaf ook wel minstens 1x per week aan dat we alles bij je kwijt konden en dat niks te gek voor je was. Ik geloof niet dat ik hier vaak gebruik van heb gemaakt omdat ik nou eenmaal niet zo'n prater ben, maar het heeft me altijd een vertrouwd gevoel gegeven. Kortom, je was een fijne pa! Je bent nu nog steeds een fijne pa, maar daarnaast ben je nu ook een fijne opa voor mijn meiden. Dat laatste is minstens zo belangrijk en helemaal niet zo vanzelfsprekend na wat er zich de laatste jaren heeft afgespeeld. Ik ben enorm trots op je grote vriend!

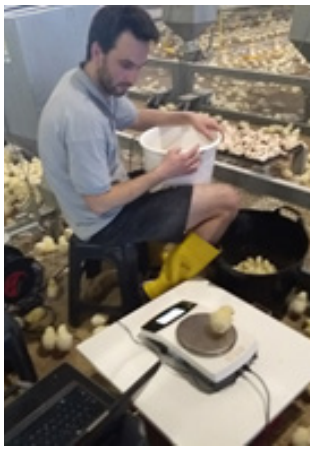
Dan nog de twee belangrijkste gasten op mijn promotiefeestje… Lieve **Madée** en **Sammie**, ook jullie bedankt! Jullie dwongen me ertoe om na het werk even uit de kuikenwereld te stappen. Bij thuiskomst sleurden jullie me letterlijk mee de trampoline op waar ik vervolgens als paard mocht fungeren. Dansen op muziek van Maan of extra hard meezingen met 'Ha ha je vader, ja je vader die is…'. Dat heeft me enorm geholpen om te relativeren, want ook al wordt een proefschrift nogal eens 'het kindje' van de promovendus genoemd, jullie doen mij beseffen waar het leven écht om draait. Sorry dan ook voor de weekenden waarin ik druk ben geweest met werken en de weken waarop ik in het buitenland was en mijn twee grote vriendinnen geen knuffel kon geven voor het slapen gaan. Wat was het dan altijd extra fijn om dit wel weer te kunnen doen bij thuiskomst met jullie enthousiasme en onvoorwaardelijke liefde. Jullie zijn nog te jong om dit nu te begrijpen, maar hopelijk lezen jullie dit prachtige boek (of alleen het dankwoord) ooit nog eens terug en vergeet dan niet om ook eventjes m'n stellingen te lezen. Dikke knuffel van een trotse papa.

"Last best" zoals men dat altijd zegt wil ik natuurlijk nog **Marisja** bedanken. Ik weet dat dit promotietraject echt niet altijd leuk voor jou geweest zal zijn. Bijvoorbeeld wanneer ik weer eens op gekke tijden moest werken en jij achterbleef met een pasgeboren ontroostbare Sammie vanwege nachtelijke krampjes. Je hebt jezelf altijd weggecijferd en deed eigenlijk nooit een beroep op me. Je gaf me daarnaast ook nog de ruimte om in mijn vrije tijd op stap te gaan wanneer ik dacht dat even nodig te hebben. Altijd zorgzaam, het hele huishouden

runnen naast je eigen baan en een topmoeder zijn voor onze meiden. Dit heeft mij ontlast en ervoor gezorgd dat ik altijd met een gerust hart van huis weg kon. Ook toonde je telkens weer je oprechte interesse als ik wat kwijt wilde over mijn werk terwijl je soms geloof ik ook net zo goed tegen de schutting had kunnen praten wanneer ik weer eens allerlei gedachtenspinsels aan het verwerken was. Door dit alles heb je heel veel bijgedragen aan mijn promotietraject, maar bovenal door me altijd te steunen en mijn lief levensmaatje te zijn. Je voelt me verdomd goed aan schat! Puur op 't gevoel…

ABOUT THE AUTHOR

Jan Wijnen was born on the 9th of March 1988. He was raised in Heesch, a village situated in the south of the Netherlands, together with his parents, grandmother, two sisters, and one brother. Jan was already intrigued by animals and especially chickens since his toddlerhood. His parents once told that little Jan often stared through the fence of their backyard chicken enclosure to imperturbably observe the chicken behaviour for minutes in a row. The first steps in 'poultry production' were taken during his teenage by slaughtering backyard chickens with his grandmother and by spending holidays helping at his uncle's poultry farm. Jan went to a secondary school in Oss at which he fell in love with Marisja since 2005. They married in 2014 and got two daughters; Madee (2015) and Sammie (2017). After secondary school, Jan studied Animal Husbandry at HAS university of applied science, 's-Hertogenbosch, the Netherlands. During this study he conducted internships on varying topics such as Q-fever in goats, milking robot capacity, st. AAP rescue center for exotic species, dairy cattle farm, and the decline of vulture populations in South Africa. After successfully completing this study he studied a master Animal Sciences at Wageningen University and Research. He conducted a minor thesis on thick fever and a major thesis on pig tail biting behaviour. After graduation he worked as a research assistant for approximately one year at the Adaptation and Physiology Group of this university. In 2017 he applied for a PhD position at this group in collaboration with HatchTech, which is a company specialized in incubation technologies. The results from this PhD trajectory are described in this thesis. Jan will continue his work on poultry incubation at the R&D department from HatchTech. for the coming years.



TRAINING AND SUPERVISION

Completed Training and Supervision Plan of graduate school WIAS

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WIAS Introduction Day	2017
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Poster – 15th European Poultry Conference (EPC) Croatia	2018
Oral - Gut Health seminar Poultry Research & Innovation Center (PRIC) the Netherlands 2019	2019
Oral – 44th Incubation and Fertility Research Group conference (IFRG) France	2019
Oral - Early nutrition seminar (Veetelers) the Netherlands	2020
Oral – 45th Incubation and Fertility Research Group conference (IFRG) E-Meeting	2021
Oral – 7th International conference on poultry intestinal health (IHSIG) Colombia	2022

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Practical Thermoregulation BSc course	2015 & 2016
Supervising Introduction to Animal Science BSc course	2015 & 2016 & 2017
BSc thesis – Marleen Verdaasdonk	2016
BSc thesis – Margriet Faber	2016
MSc major – Lara Olde Bolhaar	2018
BSc thesis – Lianne Cheung	2019
BSc thesis – Bart de Bont	2020
MSc major – Charlotte Visser	2020
MSc major – Maartje Frankena	2020
MSc minor – Iris Jansen	2021
MSc minor – Serge Alindekon	2021

Total training and supervision	**34.4 ECTS***

*1 ECTS equals a studyload of approximately 28 hours

Financial support from HatchTech B.V. for conducting the research described in this thesis and from Wageningen University for printing this thesis is gratefully acknowledged.

COLOPHON

Layout and cover by: Bregje Jaspers | ProefschriftOntwerp.nl
Printed by: ProefschriftMaken | www.proefschriftmaken.nl