ADAPTIVE CAPACITY OF REARING HENS

Effects of early life conditions

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ABSTRACT

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The traditional strategy to deal with pathogens in the layer industry is based on monitoring and control methods, primarily aimed at minimizing the risk of infection with the pathogen. The aim of this thesis was to investigate whether the adaptive capacity of layers could be influenced by early life conditions as they may occur in layer practice, as an alternative strategy for improving layer health and disease resistance. The first study investigated whether suboptimal versus optimal incubation, hatch and early rearing conditions could influence the adaptive capacity during infectious challenges with *Eimeria* and Infectious Bronchitis (IB). The second study investigated effects of prenatal high temperature manipulation on postnatal temperature preference and adaptive response of layers to heat stress. The third study investigated effects of suboptimal and optimal incubation temperature on the adaptive response to *Eimeria* under normal circumstances or following exposure to a high (35°C) environmental temperature. The fourth study investigated effects of feed provision immediately after hatch (early feeding) and suppression of gram negative intestinal bacteria (by use of the antibiotic Colistin) for 21 d post hatch on microbial composition of the intestines, layer development and response to a mix challenge with lipopolysaccharide (LPS) and humane serum albumin (HuSA). Finally, effects of early feeding and Colistin treatment on organ weights and response to an infectious challenge with *Eimeria* were investigated. Results demonstrated that optimized incubation, hatch and rearing resulted in a better adaptive response to *Eimeria* and IB, as was shown by a higher feed intake and reduced weight loss. Optimal incubation as a single early life condition also had a positive influence on the adaptive response of layers to *Eimeria*, as demonstrated by tendencies to higher feed intake and BW gain, less duodenal lesions and a lower oocyst production. Early feeding resulted in higher body and organ weights, a changed microbiota composition in the intestines, and a changed response to *E. acervulina* and LPS/HuSA. Colistin treatment resulted in a changed microbiota composition of the intestines and a changed response to *E. acervulina* and LPS/HuSA. These results confirmed the hypothesis that early life conditions can be used to influence the adaptive capacity to infectious challenges. In conclusion, improving the adaptive capacity with the use of particular early life conditions may be the first step towards an alternative method to maintain or improve layer health and disease resistance.

VOORWOORD

Op 25 augustus 2007 gingen we met een bestelbus en 2 auto's volgeladen met inboedel op weg naar een nieuw avontuur! Een hele verandering, van een 2 kamer appartement in de binnenstad van Groningen naar een ENORM huis op het terrein van Zodiac in Wageningen (wat was het hier stil 's nachts!!!), om daar op 1 september te kunnen beginnen met mijn project "Adaptatievermogen van opfokhennen". Nu, 4 jaar later, ben ik klaar! JIIEEEHAAA!!! Ik kijk terug op deze periode met een heel voldaan en trots gevoel. Natuurlijk waren er ook de nodige welbekende AIO dipjes, en "writersblocken", vooral in de laatste maanden, maar ik heb het uiteindelijk voor elkaar gekregen! Dit alles was absoluut onmogelijk geweest zonder de hulp van vele mensen, die ik bij deze wil bedanken.

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CONTENTS

GENERAL INTRODUCTION

INTRODUCTION

Layers in production systems are exposed to a wide variety of environmental challenges in which they have to perform. One of these environmental challenges is the exposure to pathogens. The traditional strategy to deal with pathogens in the animal production industry is in general based on monitoring and control methods (control model; Ten Napel et al., 2006), primarily aimed at minimizing the risk of infection with the pathogen. This control strategy should be used from the hatchery until the slaughterhouse to be most effective (Pasquali et al., 2011) and currently includes the following measures for layers: 1) biosecurity measures, which prevent the transmission of the pathogen through physical barriers (e.g. fly screens to prevent infection transmission from flies, rodent control etc.), 2) hygiene practices (e.g. protective clothing, facilities and protocols for handling hygiene, stable swabs etc.), 3) vaccination programs in the rearing period, 4) feed additives in the rearing period (e.g. for Coccidiosis prevention), 5) treatment or culling of infected or susceptible flocks dependent on the type of pathogen (virus, parasite, bacteria), severity of infestation and risk of spreading. Although the control strategies within layer production systems are effective, there are also disadvantages. First, the strategy is labour intensive, costly and very dependent on the timing and quality of the intervention, and on the protocols and equipment used. Second, vaccinations and feed additives are only used in the rearing period and aimed at certain pathogens, but do not provide full protection against all pathogenic challenges, and third, treatment after infection is often based on the use of antibiotics, which is the subject of an on-going debate in intensive animal production worldwide.

The use of antibiotics in layers (only during rearing) and other food producing animals poses risks to both animal and human health, because it is linked to the emergence of antibiotic resistant bacterial strains (Hughes et al., 2008). Transmission of these resistant bacterial strains from food animals to humans can occur after food consumption (Aarestrup et al., 2008). To reduce the risk of transmitting antibiotic resistant bacteria, the use of all antimicrobial growth promoters was prohibited at the beginning of 2006 (Regulation (EC) No. 2821/98 and Regulation (EC) No. 1831/2003). However, antibiotics are still excessively used for therapeutic purposes (FIDIN, 2010). Figure 1 shows the total use of therapeutic and growth promoting antibiotics in the animal production sector in the Netherlands from the year 1999-2010. Based on this graph and data on broilers in the report "Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2009" we expect the course of antibiotic use in poultry to be comparable (MARAN, 2009; FIDIN, 2010).

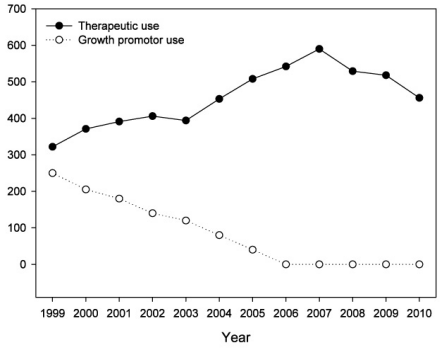

Figure 1. Veterinary therapeutic antibiotic sales in the animal production sector (cattle, poultry and pigs combined) from 1999-2009 in the Netherlands (FIDIN, 2010).

The total amount of therapeutic antibiotics has almost doubled in the period from 1999 until 2007 (Figure 1). Compared to 2007 the amount of antibiotics sold decreased with 10% in 2008, and another 2% in 2009. Recently published numbers showed an additional decrease of 12% in 2010 (FIDIN, 2010). These data indicate that producers, suppliers and consumers in the animal production sectors are more and more aware that the current approach of animal health control with spiralling use of medication is finite. This requires to search for alternatives to improve animal health and disease resistance.
A possible alternative strategy is to focus more on the animal by utilizing and supporting the intrinsic adaptive capacity for maintaining its own health and welfare during (pathogenic) challenges (adaptation model; Ten Napel et al., 2006). Animals that have a well-developed adaptive capacity are more robust and able to generate the appropriate behavioural, physiological and immunological adjustments to better maintain their health and welfare in times of pathogen exposure. In animal production, creating animals with a good adaptive capacity (robust animals) in times of challenge is rapidly becoming an important field of interest (Star, 2008).

ADAPTIVE CAPACITY

In this project the adaptive capacity was defined as the capacity to respond to an environmental stressor (e.g. pathogen) with the appropriate behavioural, physiological and immunological adjustments, in order to maintain performance, health and welfare. The development of the adaptive capacity already starts very early in life and can be influenced by several factors, for example by the genetic background, but also by perinatal development of physiological control systems, which can be affected by different environmental conditions (Figure 2).

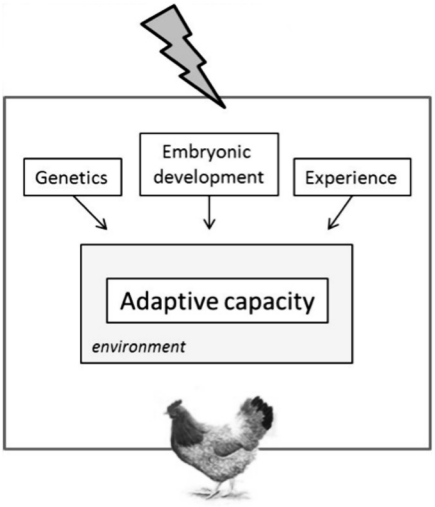

Figure 2. The adaptive capacity of an animal is important in the response to a stressor and is influenced by genetics, embryonic development and early life experience. The environment of the animal should be supportive enough to allow full expression of the adaptive capacity.

If we aim at influencing the adaptive capacity of animals in commercial circumstances, a layer is a good model, because both embryonic (in ovo) and post hatch development can be influenced by environmental conditions without any interference of the mother, which is much more difficult in animals that develop in

utero. It is therefore relatively easy to investigate if and how management practices can influence the adaptive capacity of layers in later life.
The genetic background of a layer is fixed and cannot be changed only by management. However, it is possible to manipulate both pre- and early postnatal conditions in layers, affecting the development of the adaptive capacity within the given genetic background. In this way, layers with a less optimal life history are still able to perform well in a certain environment, because the early life conditions they experienced prepared them to life in that particular environment (epigenetic adaptation; Dorner, 1974). Thus, when the early environment matches the later life (expected) environment, this is beneficial for the performance and the adaptive capacity of a layer in that particular environment. On the other hand, if there is a mismatch between the early and later life environment, this will result in poorer performance and even maladaptation (Schmidt, 2011; Figure 3).

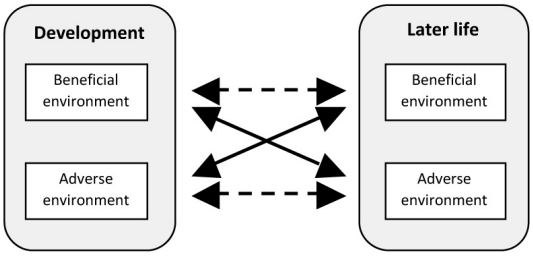

Figure 3. Representation of the match/mismatch hypothesis (adapted from Schmidt, 2011). If the environment of the animal during pre- and/or early postnatal development matches the
environment in later life (dashed arrows), this is beneficial for the performance and adaptive capacity of the animal in that particular environment. In contrast, a mismatch between the early and later life environment (full arrows) will result in maladaptation and poor performance of the animal.

In order for a layer to express and utilize its adaptive capacities to the full extent, the current environment should also be supportive enough in all its resources in order for (ten Napel et al., 2006). For layers and other production animals, the environment can be a limiting factor in the expression of their adaptive capacity. This is mainly because the design of current production systems is not based on the idea that animals should be able to use their own adaptive capacities in times of environmental challenges, but more on creating the optimal environment for high production levels using the above described control strategy. For example, if a layer in a free range housing system experiences a too high ambient temperature, for example during summer, and wants to adapt by seeking a cooler spot, this is impossible when the housing system does not provide shelter possibilities. In this particular case, the environment lacks the support necessary for the layer to adapt (ten Napel et al., 2006).

THE IMPORTANCE OF EARLY LIFE CONDITIONS

Incubation

Layers develop in ovo where embryos can already be influenced by environmental conditions. The most important environmental factor for a layer embryo to start development is incubation temperature (Decuypere and Michels, 1992; Meijerhof, 2009; Molenaar et al., 2010a,b). The optimum incubation temperature of chicken eggs ranges between 37°C and 38°C (Wilson, 1991) and deviations from this optimum incubation temperature can have negative effects on embryo development (Romanoff, 1972; Shafey, 2004; Molenaar et al., 2010a,b), hatchability (Deeming and Ferguson, 1991; Decuypere and Michels, 1992), chick quality (Lourens et al., 2005; Molenaar et al., 2010a,b) and subsequent performance (Decuypere, 1979; Lourens et al., 2005). Indirectly, incubation temperature may also affect the capacity to adapt during pathogen exposure. For example, Molenaar et al. (2010a) demonstrated that high incubation temperature (38.9°C) in the last week of incubation resulted in chickens with reduced organ weights, including the Bursa of Fabricius. Moreover, Oznurlu et al. (2010) found that the normal embryonic development of the Bursa of Fabricius was significantly retarded after high incubation temperature from embryonic day 10-21. The Bursa of Fabricius is the primary organ for B-cell development in chickens and a smaller bursa may result in a lower antibody production (Giannessi et al., 1992), which can be a disadvantage in times of a pathogen encounter.

For the poultry industry it is important that eggs are incubated under optimal incubation temperature, in order to ensure good development, performance and health of the chickens in later life. The optimum incubation temperature is based on maximum hatchability and good chick quality. Lourens et al. (2005) showed that an eggshell temperature of 37.8°C gave the best results in broilers. However, it has proven to be difficult to maintain a constant incubation temperature in artificial incubation. In large commercial incubators air temperature is often used as the treatment applied to the eggs (French, 1997). However, Meijerhof and Van Beek (1993) demonstrated that broiler embryos frequently become overheated during artificial incubation, even when the incubator temperature is set in the optimal range. This is because the embryo experiences a different temperature than the temperature in the incubator, which is related to the heat production of the embryo and the heat transfer between the egg and its surroundings (Meijerhof and Van Beek, 1993; Lourens et al., 2011). During early incubation, the embryonic temperature is close to, but a little bit lower than the incubator temperature, because of evaporative cooling of the egg. From mid incubation onward, the embryo is growing rapidly and its metabolic heat production increases. Consequently, the embryo temperature will now raise above incubator temperature and thus above the optimum incubation temperature as well (French 1997). Because it is not possible to measure the embryo temperature without disturbing the embryo, the egg shell temperature (EST) is often used as an indicator of embryo temperature (Lourens, 2001). Eggshell temperatures only deviate from embryo temperatures by 0.1-0.2°C (Meijerhof and Van Beek, 1993). Previous studies demonstrated that a continuous EST between 37.5 and 38.0°C during the first 18 days of incubation results in the highest hatchability and best performance post hatch (Lourens et al., 2005, 2007). In order to obtain the best results in practice, according to these studies in broilers, the incubator temperature should therefore be adjusted to maintain a constant EST between 37.5 and 38.0°C. Whether

these incubation temperatures are, besides hatchability and performance, also the most ideal for the development of the capacity to adapt to environmental challenges was, to our knowledge, never investigated. Although large deviations from optimal incubation temperature seem to have a negative effect on the growing embryo and hatchling, more and more studies in broilers and ducklings report that higher or lower EST during certain `critical periods' of embryo development might result in epigenetic adaptation, i.e. the adaptation of an animal to an expected environment (Nichelmann, 1992). These critical periods can usually be defined as periods in which important physiological systems develop and mature, e.g. the thermoregulatory system and HPA-axis. Some functional systems are maturing earlier in embryonic development, others later (Decuypere and Michels, 1992). During these so-called critical periods of functional development, the environment experienced by the embryo can predetermine the actual set-point of these control systems for the entire post hatch life (Dorner, 1974; Nichelmann et al., 1999; Tzschentke and Plageman, 2006). Manipulations in incubation temperature as an environmental factor can result in epigenetic adaptation, but it depends on the timing, level and duration of the temperature manipulation (Yahav et al., 2009). For instance, chickens exposed to high incubation temperature during mid and late embryogenesis (ED 14-21) can better adapt to high environmental temperatures post hatch (Tzschentke, 2007, 2008; Yahav et al., 2009). However, adaptation to an environment often has a trade-off and it is likely that chickens programmed for a particular environment, may be less flexible to respond if the environment suddenly changes or if they are exposed to other environmental challenges (Schmidt, 2011). In this way, incubation temperature may also a-specifically influence the adaptive capacity of chickens to different environmental challenges, like pathogens. The fact that incubation temperature can easily be manipulated in practice, makes it a useful tool to investigate the possibilities of influencing the adaptive capacity of layers during different environmental circumstances, which may ultimately benefit their production, health and welfare.

Rearing

During the first days of their life, chickens are most susceptible to pathogens, including those common in the environment of poultry facilities. This susceptibility decreases as the bird matures, suggesting that this phenomenon might be explained by an immaturity of the immune system during the first 1-2 weeks after hatching (Lowenthal et al., 1994). Dibner et al. (1998) showed that the immune system of the chicken is only partly developed at hatch. The primary immune organs, the thymus and the bursa, are both present and already populated by lymphoid tissue (Dibner et al., 1998). However, the immune system is not able to respond properly to antigens yet. In the first few days post hatch, chickens depend on innate and maternal immunity and slowly transit to a dependence on endogenous adaptive immunity (Butler et al., 2006). IgA is the most important immunoglobulin in the intestinal mucosal immune system of chickens and is held responsible for the defense against pathogenic micro-organisms (Macpherson et al., 2008). Lammers et al. (2010) demonstrated that endogenous IgA is almost undetectable before 14 days of age, but mRNA expression levels of IgA increase rapidly from d 21 of age. Moreover, maternal IgA has disappeared when the chicken reaches the age of 14 days. This means that around 14-21 days of age there is a so called 'IgA gap', in which penetration of bacteria of the mucosa may be enhanced, which is important for further immune development.

This suggests that environmental factors in early life might influence the further development of the system and could therefore also affect the adaptive capacity of the chickens to future pathogenic challenges. Important environmental factors could be the early access to feed and water after hatching, the ambient temperature during the first days after hatching and the enrichment of the housing facilities.

Early Feed Availability

Due to the hatching window in commercial hatcheries, some hatchlings might spend up to 36-48 hours without access to feed and water, which causes poor viability and retarded growth. In contrast, when feed is immediately provided after hatch, (broiler) chickens have higher growth rates, improved intestinal development, and a better development and maturation of the intestinal (Gut Associated Lymphoid Tissue; GALT) and systemic immune system (Noy et al., 2001; Juul-Madsen et al., 2004; Bar-Shira et al., 2005). Dibner et al. (1998) investigated the effects of early feeding (feed provision immediately after hatch) on immune development and the response to an oral challenge with coccidial oocysts. Overall, early feeding positively influenced the development of the immune system, which was indicated by heavier bursa weights and earlier appearance of IgA and germinal centres. IgA is a part of the mucosal immune system and is the last of the major isotypes to appear. The presence of IgA is a sign that the humoral immune system is fully developed. Furthermore, the response to the coccidial challenge was also improved, as fed chickens had a higher body weight than non-fed chickens after challenge.

The positive effects of early feeding might be related to the microbiota composition in the chicken intestine. Colonization of the chicken gut starts immediately after hatch (La Ragione et al., 2005) and feed is a source of many (harmless) bacteria. The earlier the feed passes through the intestinal tract, the sooner the epithelial surface of the intestinal mucosa comes into contact with these bacteria (Uni et al., 1998). As dendritic cells of the immune system continuously sample the gut, early exposure to these bacteria through the feed may help to create a wider antibody repertoire (Uni et al., 1998). Feed also provides a substrate for bacteria already present in the gut lumen and thereby early feeding has the potential to influence bacterial composition and successive colonization. The composition of the gut microbiota in early life is therefore very important in development of proper immune functionality in later life (Amit-Romach et al., 2004) and it can easily be envisaged that changes in the gut microbial composition of chickens affect the immune functionality and thereby also the adaptive capacity to an intestinal pathogen later in life. Although the results of the previous performed experiments on early feeding look promising for the development of more robust layers, there are also some discussion points. First, almost all studies were performed in broilers and second, these studies only investigated short term consequences (< 14 days) of early feeding on immunity, performance and the ability to respond to a pathogenic challenge. It is possible that the provision of feed immediately post hatch may also have (longer lasting) effects in layers, but this has hardly been investigated.

Temperature

Short-term thermal manipulation during the early post natal phase can induce thermo tolerance in later life. Broiler hatchlings exposed to 36°C for 24h at 5 days of age had

a higher ability to cope with heat stress (35°C for 1 h) at 42 days of age (Yahav and Plavnik, 1999) and lower heart weights, haematocrit value and plasma triidothyronine (T3) levels (Yahav and Hurwitz, 1996). Besides thermal manipulation, housing temperature is also important for performance later on. Broiler chickens reared in a colder environment (34 vs. 29°C) in the first 6 days of life, had a lower body weight at slaughter age (d 35) than broiler chickens raised at the normal practice temperatures (Baarendse et al., 2006).
These studies demonstrate that housing temperatures in the early post-natal phase seem to have long term effects on performance and thermo tolerance of broilers. This indicates that there is a direct effect of the early life temperature exposure on the response to a temperature stress in later life. Although this field of research seems to be well covered in broilers, there is very little knowledge about these effects in layers. Furthermore, whether there is a more a-specific effect of early life temperature exposure, e.g. on the performance and the capacity to adapt to challenges, has never been investigated.

Environmental Enrichment
Environmental enrichment can be defined as modification of the animals' environment that enhances behavioural opportunities and leads to an improvement of biological functioning (Newberry, 1995). The benefits of enrichment to chickens are numerous and include encouraging a more-even distribution of animals in the pen (Cornetto and Este´vez, 2001), reducing disturbances and aggression (Cornetto et al., 2002), and reducing fear responses and stress (Jones, 1982; Nicol, 1992; Reed et al., 1993; Grigor et al., 1995; Bizeray et al., 2002). Enrichments that promote foraging seem to reduce aggression and feather pecking in chickens (Gvaryahu et al., 1994; Huber-Eicher & Wechsler, 1998). Providing poultry with structural complexity by creating functional units in their pen with nests, perches and foraging substrate like peat dust can increase activity levels and allows the birds to control their social interactions like aggression (Bizeray et al., 2002). Besides increasing social behaviour and activity levels in chickens, substrates like peat dust is also important for the maturation of the immune system, as is demonstrated in a human study. Peat dust contains harmless bio particles (bacteria, molds and fungal spores) that trigger the immune system without causing any overt reaction (Hauswirth, 2004; Hauswirth and Sundy, 2004). Without sufficient exposure to these bio particles, the immune system might not mature properly, which increases the risk for developing asthma and atopy in humans. By providing substrates, like peat dust, in poultry pens in practice we might contribute to the maturation of the chickens' immune system. Whether enrichment of the environment also influences the adaptive capacity during environmental challenges has yet to be investigated.

AIM AND OUTLINE OF THE THESIS

The project "Adaptive capacity of rearing hens; effects of early life conditions" focuses mainly on the adaptive capacity of young layers in times of infectious challenges. The rearing period (<17 wk) is an important period as it prepares the hens for the production period (>17 wk), in which they are expected to be well developed, healthy and lay as many as 300 eggs a year. It is therefore important that layers develop well in the rearing period and are already able to withstand environmental challenges. Therefore, the focus of this thesis was to influence the adaptive capacity of layers in the rearing period.

The first aim of the thesis was to investigate whether contrasts in incubation, hatch and early rearing conditions as found in layer practice could influence the adaptive capacity during infectious diseases **(Chapter 2)**. In order to do so, the intestinal parasite *Eimeria* (responsible for the disease Coccidiosis) and the Infectious Bronchitis virus (IB), were chosen as model infections to investigate the principle. Both diseases are frequently observed in poultry production systems, where they spread rapidly due to the high stocking densities. To measure the adaptive capacity after the infectious challenges, several physiological and immune parameters were measured, for example body weight (gain), feed intake, IB antibody titre and *Eimeria* oocyst production. The **Chapters 3-6** looked more into detail at particular early life conditions and their consequences for the adaptive capacity. **Chapter 3** describes a pilot experiment to determine whether layers were sensitive to thermal manipulation during embryogenesis and whether this could be used to influence their adaptive capacity to high environmental temperatures and thereby decrease their temporary susceptibility to other challenges. **Chapter 4** investigated the effects of suboptimal incubation temperature on the adaptive response to *Eimeria*. Moreover, the stress load at the moment of infection was varied by exposing chickens to a high environmental temperature preceding the infectious challenge. The idea behind this approach was that well adapted layers should be flexible enough to respond properly to an infectious threat even if they are under stress conditions.

The effects of early feeding and antibiotic treatment on intestinal microbiota changes and response to a model lung infection with LPS/HuSA are presented in **Chapter 5**, whereas the effects of early feeding and antibiotic treatment on layer development and adaptive capacity to an infectious challenge with *Eimeria* acervulina are described in **Chapter 6**. The major findings in **Chapter 2-6** will be discussed in the General Discussion **(Chapter 7)**.

EARLY LIFE EXPERIENCES AFFECT THE ADAPTIVE CAPACITY OF REARING HENS DURING INFECTIOUS CHALLENGES

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ABSTRACT

The present study aimed to investigate whether pre- and early postnatal experiences of rearing hens contribute to the ability to cope with infectious challenges at an older age. In a 2 x 2 factorial arrangement, 352 Lohmann Brown chicks were exposed to either suboptimal or optimized incubation plus hatch conditions and to cage or enriched rearing from wk 0-7 of age. After wk 7 all rearing conditions were similar until the end of the experiment. The development of adaptive capacity to infectious challenges was investigated by introducing an *Eimeria* and Infectious Bronchitis (IB) infection on d 53 and d 92, respectively. Body weight gain and feed intake during the infections, duodenal lesions and amount of positive stained $CD4^+$ T cells, $CD8^+$ T cells and macrophages at d 4 and 7 after *Eimeria* infection, as well as the IB antibody titre throughout the experimental period were determined.

The results demonstrated a significant interaction between incubation plus hatch and rearing environment. Optimized incubation plus hatch conditions followed by an enriched rearing environment resulted in the least weight loss ($P<0.05$) and the highest feed intake ($P<0.01$) from d 3 until 7 after the *Eimeria* infection (d 56-60 of age), compared with all other treatments. In addition, the optimized x enriched chicks had the highest BW gain from d 7 until 14 after IB infection (d 99-106 of age), compared to chicks housed in a cage environment ($P<0.01$). Besides the interaction, optimized incubation plus hatch alone resulted in reduced macrophage numbers in the duodenal tissue at d 4 after *Eimeria* infection, compared to suboptimal incubation plus hatch, whereas the enriched rearing environment stimulated the recovery of intestinal damage caused by *Eimeria* ($P<0.05$) and reduced the production of specific antibodies after IB infection ($P<0.05$), compared to the cage environment. In conclusion, the present study shows that early life experiences can indeed affect the capacity of rearing hens to cope with an *Eimeria* and IB infection at an older age, in which performance of chicks is best maintained after optimized incubation plus hatch followed by enriched rearing. This suggests that the development of adaptive capacity to infectious challenges can be influenced with management during a short period in pre- or early postnatal life, but that effects last for a considerable time after cessation of the specific management.

Keywords: early life conditions; adaptation; laying hens; infectious diseases

INTRODUCTION

Rearing hens in production systems are exposed to different challenges. One of these challenges is the exposure to various infectious agents. The traditional strategy to reduce the occurrence and spread of infectious agents in layer flocks is based on prevention management, by using feed additives, hygiene measures, vaccination programs and medication (Reid, 1989; Van Immerseel et al., 2002). Although it has proven to be effective, prevention management also has disadvantages. Extensive use of vaccinations and medication will increase the production costs for the farmer as well as the risk for development of more resistant pathogens. An alternative strategy for prevention management is to increase the adaptive capacity of laying hens to cope with infectious challenges. By increasing the adaptive capacity, laying hens will be more able to combat infections before they spread to the flock, while maintaining their performance and welfare at a sufficient level.

The development of an animal's adaptive capacity does not only have a genetic component, but is also influenced by experiences in pre- and early postnatal life (Star, 2008). Previous studies in different animal species (Vallee et al., 1997; Vanbesien-Mailliot et al., 2007; Merlot et al., 2008) have demonstrated that environmental changes and stressors during certain critical periods in pre- and early postnatal life might exert a major impact on the development of important functional systems, including the immune system. The majority of these studies have been conducted in mammals, but there are also a few studies in poultry. For instance, Janczak et al. (2006) simulated prenatal stress in chickens by an injection of corticosterone in the eggs. Chicks exposed to prenatal corticosterone were more fearful than controls after hatch, which is often considered as maladaptive behaviour. Another example is given by Piestun et al. (2009). The ability of broilers to cope with heat stress at market age can be influenced by thermal conditioning to high temperatures during embryonic development. In addition, and also important for the focus of the current study is that the development and functioning of the immune system as well as disease resistance in poultry can be stimulated by early feed provision directly after hatch (Dibner et al., 1998; Bar-Shira et al., 2005), but also by early handling in the first 10 d post hatch (Huff et al., 2001). The above mentioned studies demonstrate that a variety of functional physiological systems, that are important for adaptation to challenges, can be affected by early life experiences. Laying hens in production systems vary substantially in their early life experiences due to differences in incubation, hatchery and rearing management. For proper functioning of the total production chain of laying hens, it is important to know whether and to what extent these differences in management systems and thereby in early life experiences, might affect the development of adaptive capacity to infectious challenges in the chicks.

The aim of the current study was therefore to determine whether differences in early life experiences during pre- and early postnatal life of chicks (< 8 weeks) can result in differences in the chicks' capacity to adapt to infectious challenges later on (>8 weeks). Based on the knowledge from previous studies, we hypothesized that it would indeed be possible to create differences in adaptive capacity due to different early life experiences. It was expected that an optimized incubation and hatch environment will reduce perinatal stress and will ultimately result in better developed chicks that will be able to cope with challenges later in life. Furthermore, we hypothesized that a post natal environment that stimulates the expression

of natural behaviour will also have positive effects on the development of the chicks and their adaptive capacity.

MATERIALS AND METHODS

All experimental protocols were approved by the Animal Use and Care Committee of the Animal Sciences Group, Lelystad, the Netherlands.

Incubation plus hatch

Eggs (Lohmann Brown; flock age 42 wk, Verbeek Hatchery, Lunteren, The Netherlands) were randomly assigned to one of two treatments, suboptimal or optimized incubation plus hatch. Incubation occurred in two different climate respiration chambers (CRC), one for each treatment (Lourens et al., 2006). Egg shell temperature (EST) was used as treatment applied to the eggs, as a reflection of embryo temperature (Lourens, 2001).

The suboptimal incubation plus hatch treatment (S) was based on conditions that occur in practice. In commercial hatcheries, a relatively constant incubator temperature (± 37.8°C) is often used as treatment applied to the eggs. However, there is an important difference between the incubator temperature and the actual embryo temperature. Due to the imbalance between embryonic heat production and (latent) heat transfer in early and late incubation, the embryo temperature during early incubation is lower than the incubator temperature. On the other hand, a higher embryo temperature compared to incubator temperature is observed at the end of incubation, when the embryo is producing heat itself (French, 1997; Hulet et al., 2007). The aim of the suboptimal incubation plus hatch treatment (S) was to mimic embryo temperature during incubation and conditions after hatch in commercial hatcheries (especially multi-stage incubators). Therefore, eggs (n=336) were exposed to a low EST (36.7°C) in wk 1, an optimal EST (37.8°C) in wk 2 and a high EST (38.9°C) in wk 3 of incubation. Relative humidity was between 50 and 55%. On embryonic day (ED) 19, all eggs were placed in hatching baskets. On ED 20 and 21, dry hatchlings of both sexes were collected every 3 h and body weight (BW) and chick length (Hill, 2001; Molenaar et al., 2008) were measured in the CRC. To simulate post hatch conditions in commercial hatcheries, hatchlings were returned to their hatching baskets and remained in the CRC (ambient temperature (T_a) = 38°C and lights off) until all eggs within the treatment had hatched.

In the optimized incubation plus hatch treatment (O), 336 eggs were incubated at a constant EST of 37.8°C, which is considered as the optimal EST for chicken eggs (Lourens et al., 2005). Relative humidity (RH) was set between 50 and 55%. On ED 19, all eggs were placed in hatching baskets. On ED 20 and 21, dry hatchlings of both sexes were collected every 3 h and BW and chick length was measured in the CRC. To optimize post hatch conditions, hatchlings were transported to another CRC with a more comfortable ambient temperature (T_a=34°C, 60-65% RH, 24 h light) and had unlimited access to feed (175 g/kg crude protein, 11.31 MJ ME/kg, ileal digestible lysine: 0.37%), water and foraging material. In addition, chicks from the optimized incubation plus hatch treatment had a heating lamp available in their environment from hatch until d 10 of age to provide additional warmth if necessary.

Rearing environment

Before transportation to the rearing environment (referred to as d 1), all hatched chicks were vaccinated against Infectious Bronchitis (IB) (Nobilis® IB Ma5, Intervet, Boxmeer, the Netherlands) and Newcastle Disease (Nobilis® ND Clone 30, Intervet, Boxmeer, the Netherlands). Thereafter, 176 chicks from both incubation plus hatch treatments were randomly selected and equally distributed over two rearing environments, which resulted in a 2 x 2 factorial arrangement (Figure 1).

The cage environment (C) consisted of wired cages (75 x 65 cm), with the wires covered in the first 10 d of rearing to prevent foot damage. The enriched rearing environment (E) consisted of floor pens (100 x 75 cm) with wood shavings, peat dust and perches. Chicks from both cage and enriched environments had unlimited access to feed and water in storage feeders and drinking cups. Storage feeders had the same width as the cage/pen and there was enough space to allow simultaneous feeding of all chicks. Commercial phase 1 diet (175 g/kg crude protein, 11.31 MJ ME/kg, ileal digestible lysine: 0.37 %) was fed during the first 7 weeks of rearing and commercial phase 2 diet (160 g/kg crude protein, 11.31 MJ ME/kg, ileal digestible lysine: 0.31%) from week 7 until the end of the experiment. Both rearing environments consisted of 16 cages/pens and 11 chicks were housed per cage/pen. The Ta was 30°C at d 1 and d 2 and declined with 1°C every 3 days until 20°C at d 36 and the remaining weeks of the experiment. At d 48 of age, the rearing environment contrast was ended and all chicks were housed in floor pens, with wood shavings and perches until the end of the experiment (d 115 of age).

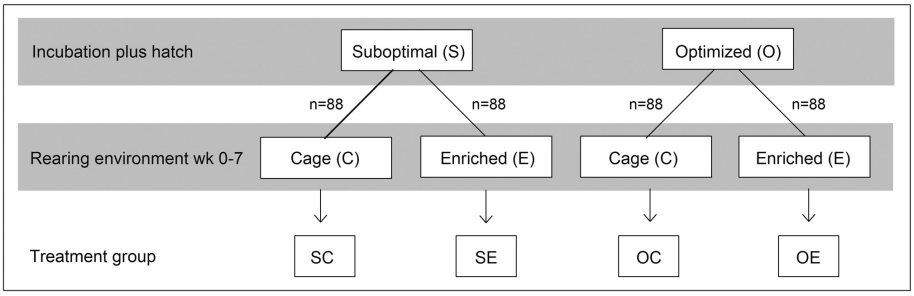

Figure 1. Experimental design and treatment groups.

Infectious challenges

A PBS solution containing 25,000 sporulated *Eimeria acervulina*, 5,600 E. maxima and 3,100 *E. tenella* oocysts (Animal Health Service, Deventer, the Netherlands) was used for the infection. At d 53 of age, all chicks were inoculated in the crop with 1 mL of the mixed *Eimeria* species solution. To determine the dose, a pilot study was conducted with different compositions of *Eimeria* subspecies. At 4 and 7 days after inoculation, the intestines were scored for lesions. The severity of the lesions ranged from 0 (no lesions) to 4 (severe lesions) (Animal Health Service, Deventer, the Netherlands). The optimal dose was based on an average lesion score of 2, with a variation between score 0 until 4, but was not severe enough to cause mortality among chicks. At d 92, all chicks were inoculated in the nostrils with a 1 mL solution (10^4 EID50, Animal Health Service, Deventer, the Netherlands) of the same IB virus strain they

were vaccinated against. The aim of the IB challenge after vaccination was to investigate differences in the adaptive (humoral) immune response by determining IB antibody production.

Embryonic mortality and hatchability
At d 7 and 19 of incubation, eggs were candled and infertile eggs or eggs containing dead embryos were removed from the CRC. After completion of the hatching process, the remaining un-hatched eggs were also removed from the CRC. All removed eggs were visually examined to determine true fertility or moment of mortality as described by Lourens et al. (2006).

Body weight and feed intake
Body weight (BW) was recorded individually at d 1, 15, 22, 36, 50, 56, 57, 60, 64, 83, 96, 99 and 106. Feeders were weighed to determine feed intake on pen level at d 1, 8, 15, 22, 36, 48, 50, 53, 54, 55, 56, 57, 60, 61, 64, 71, 83, 95, 96, 97, 98, 99 and 106. The frequency of both BW and feed intake measurements was higher during the *Eimeria* (d 50-64) and IB (d 95-99) infection.

Blood and tissue sampling
Six chicks per cage/pen were used for blood sampling. The same chicks were used for all samplings. For plasma corticosterone measurements, four of these chicks per cage/pen were sampled from a wing vein at d 14, 21, 46, 57 and two of these four chicks per cage/pen at d 60. Blood was centrifuged at 3,000 rpm for 15 min to obtain plasma. Plasma samples were stored at -20°C for further analysis by a time-resolved fluoroimmunoassay (TR-FIA) (De Jong *et al*., 2001). The other two of the six sampling chicks per cage/pen were used for IB titre determination. Blood was drawn from the wing vein at d 14, 21, 34, 91, 98, 106 and left to coagulate overnight at room temperature. Serum was stored at -20°C for IB titre analysis with a Hemagglutination Inhibition Assay (De Wit, 2000).
At d 4 and 7 after the *Eimeria* multi-species infection, one chicken from each pen was dissected and the duodenum, jejunum and caeca were scored for lesions. The severity of the lesions ranged from 0 (no lesions) to 4 (severe lesions) (Animal Health Service, Deventer, the Netherlands). A section of the duodenum, jejunum, caeca and spleen (1 cm) was collected, snap frozen under liquid nitrogen and stored at -80°C for immunohistochemical analysis.

Immunohistochemistry
Immunohistochemical staining was performed by an indirect immunoperoxidase staining (Nakane, 1975) on frozen tissue sections (8μm) of duodenum and spleen, collected at d 4 and 7 after *Eimeria* inoculation. Endogenous peroxidase was inhibited by 2% NaN_3 in TRIS-HCL 0.05 M (pH 7.5) + 0.06% H_2O_2 for 5 min at room temperature. Slides were subsequently incubated for 30 min with monoclonal antibodies against $CD4^+$ T cells (1:400; CT-4 Southern Biotech, Birmingham, U.S.A), $CD8^+$ T cells (1:400; CT-8, Southern Biotech) and macrophages (1:100; KUL, Southern Biotech). Second antibody incubation occurred with peroxidase-conjugated rabbit anti-mouse Ig (1:100; P161, Dako, Glostrup, Denmark) for 30 min. Peroxidase activity was detected by 0.05% 3,3-diaminobenzidine (DAB) in 0.1 M TRIS-HCL (pH 7.5) with 0.03% H_2O_2. After counterstaining with haematoxylin, two images of the duodenum and spleen

were acquired per chicken with a Nikon Microphot FXA microscope (Nikon Instruments Europe BV) with an Olympus DP50 camera and analysed with the software AnalySIS (version FIVE, Olympus B.V.). The colour thresholds of the software were adjusted to either match the colour of stained or unstained cells. After this step, an area of 1 μm^2 tissue was selected in duodenum and spleen and the area of stained and unstained cells was calculated by using the colour thresholds.

Tonic Immobility test

At d 47 of age, two animals per cage/pen were subjected to a Tonic Immobility (TI) test (Jones et al., 1994) to investigate fear related behaviour. A manual restraint was applied to induce TI, by placing the chick on its back and restraining it with one hand over the sternum for 10 seconds. If TI could not be induced in the first restraint, the procedure was repeated until a maximum of 4 restraints to induce TI. If TI could not be induced, the score was 0 sec. When induction was successful, the duration was recorded for a maximum of 300 sec. The recording ended after the chicken righted itself, or when the time limit was reached. In the latter situation, the score given was 300 sec.

Statistical analysis

The data were analysed as a 2 x 2 factorial design with two incubation plus hatch treatments and two rearing environments. When means and residuals were not normally distributed, the data was log transformed before analysis. All analyses were performed with SAS software (SAS 9.0, SAS Institute Inc.) Embryonic mortality and hatchability were analysed with a logistic regression procedure (proc logistic) with incubation plus hatch treatment as class variable. Egg was the experimental unit.

Body weight and chick length at hatch were analysed using generalized linear regression (proc glm) with incubation plus hatch treatment as class variable. For chick length, the person measuring was added to the model as class variable. Chick was used as experimental unit.

Both body weight and feed intake throughout the experimental period, as well as corticosterone levels and IB titre were averaged per cage/pen and analysed with a generalized linear regression for repeated measurements (proc mixed). Cage/pen was included as the repeated subject and the (AR(1)) covariance structure was the best fit. Incubation plus hatch treatment, rearing environment, age and their interactions were included as class variables. Percentage of males per cage/pen was added as covariate to the body weight and feed intake model, to correct for effects due to the unequal distribution of the sexes over treatments. Non-significant interactions ($P>0.05$) with age were excluded from all four models. The experimental unit was cage/pen.

Body weight gain during *Eimeria* and IB infection was determined for each individual chicken, but was subsequently averaged per cage/pen. Analysis was performed by a generalized linear regression (proc glm). Incubation plus hatch treatment, rearing environment and their interaction were class variables in the model. Percentage of males per cage/pen was added as covariate to correct for effects due to the unequal distribution of the sexes over treatments. Cage/pen was used as the experimental unit.

Intestinal lesion and TI induction data were analysed by a logistic regression procedure (proc logistic) with incubation plus hatch treatment, rearing environment and

their interaction as class variables. TI duration and immunohistochemistry data were analysed using generalized linear regression (proc glm) with incubation plus hatch treatment, rearing environment and their interaction as class variables. Chicken was the experimental unit. Data are expressed as means ± s.e.m.

RESULTS

Embryonic mortality, hatchability and chick quality

Suboptimal incubation treatment tended to increase embryonic mortality in the first week by 4.34 % (*P*=0.06) (Table 1). Embryonic mortality in the last week of incubation was 5.94 % higher in the suboptimal than in the optimized incubation treatment (*P*=0.02). The suboptimal incubation treatment increased the incubation time with 10 h (*P*<0.001) and resulted in a 9.00% lower hatchability (*P*=0.001) compared with the optimized incubation treatment. At hatch, chick length tended to be shorter in suboptimal incubated chicks (*P*=0.09), but there was no difference in hatching body weight between incubation treatments (*P*=0.97).

Table 1. Effect of suboptimal and optimized incubation treatment on embryonic mortality, hatchability, incubation time, body weight and chick length at hatch. Values are percentages or mean ± s.e.m.

	Treatment[1]		
Item	Suboptimal	Optimized	P-value
Embryonic mortality (%)			
wk 1	11.38	7.04	n.s.
wk 2	1.23	1.53	n.s.
wk 3	11.08	6.12	*
Hatch of fertile (%)	76.3	85.3	**
Incubation time (h)	501.0 ± 0.3	491.0 ± 0.3	***
Body weight (g)	43.9 ± 0.2	44.1 ± 0.2	n.s.
Chick length (cm)	17.8 ± 0.03	17.9 ± 0.03	n.s.

[1]Suboptimal = eggshell temperature (EST) of 36.7°C in wk 1, 37.8°C in wk 2 and 38.9°C in wk 3 of incubation; Optimized = EST of 37.8°C in wk 1, 2 and 3 of incubation.

*P<0.05; **P<0.01; ***P<0.001; n.s.= not significant

Body weight and feed intake

During the experimental period

No interaction was observed between the main effects incubation plus hatch treatment and rearing environment for BW and feed intake. Until d 22, chicks from the optimized incubation plus hatch treatment were heavier than the suboptimal incubation plus hatch chicks (an average of 129.7 ± 1.3 g vs. 123.3 ± 1.7 g in this period, respectively) (*P*=0.0002). No difference in BW between incubation plus hatch treat-

ments was observed after d 22. In addition, no difference in feed intake between incubation plus hatch treatments was observed over the experimental period.
Chicks reared in the enriched environment had a higher BW than cage reared chicks at d 15 of age (136.9 ± 1.49 g vs. 131.5 ± 1.35 g), whereas the opposite was observed at d 36, 50, 56, 83 and d 96 of age (rearing environment x age interaction, *P*=0.02). In addition, chicks reared in an enriched environment had a higher average feed intake (66.77 ± 1.12 g/d) compared to chicks reared in a cage environment (63.55 ± 1.03 g/d, *P*=0.05) over the entire experimental period.

During Eimeria infection
Chicks were inoculated with *Eimeria* at d 53 of age. BW gain and feed intake were determined from d 50-56, d 56-60 and d 60-64 of age (Table 2). Chicks that received the optimized incubation plus hatch treatment and were subsequently reared in an enriched environment lost the least weight and had the highest feed intake from d 56-60, compared with all other treatments (incubation plus hatch x rearing environment interaction, *P*=0.01 for BW and *P*=0.005 for feed intake). The effect of rearing environment was significant for BW gain and feed intake during infection from d 50-56. Enriched reared chicks lost 10.7 g less body weight (*P*=0.03) and had a slightly higher (+ 22.7 g) feed intake (P=0.06) than cage reared chicks. The higher feed intake in enriched reared chicks maintained during the period of d 60-64 (*P*=0.005).

Table 2. Effect of suboptimal and optimized incubation plus hatch, followed by a cage or enriched rearing environment in week 0-7, on body weight gain and feed intake during a multi-species *Eimeria* infection*. Values are mean ± pooled s.e.m.

		Body weight gain (g)			Feed intake (g/chick)		
Incubation + hatch	Rearing	d 50-56	d 56-60	d 60-64	d 50-56	d 56-60	d 60-64
Suboptimal	Cage	146.6	-55.2[a]	82.3	415.4	167.8b	291.6
Suboptimal	Enriched	157.2	-58.3[a]	79.3	455.3	155.6b	307.7
Optimized	Cage	141.7	-52.2[a]	85.6	405.9	153.8b	275.3
Optimized	Enriched	150.1	-28.1[b]	79.1	437.8	211.5a	329.2
Pooled s.e.m.		2.9	4.8	3.9	13.4	9.0	9.2
P-value		n.s.	*	n.s.	n.s.	**	n.s.

[a,b] Within a column, different superscript letters indicate significant differences at *P*<0.05.

*Chicks were inoculated in the crop at d 53 of age with a 1 mL PBS solution containing sporulated oocysts of *E. acervulina* (25,000), *E. maxima* (5,600) and *E. tenella* (3,100).

**P*<0.05; ***P*<0.01; ****P*<0.001; n.s.= not significant

During IB infection

Chicks were inoculated with IB at d 92 of age. BW gain and feed intake were determined from d 83-96, d 96-99 and d 99-106 of age. No treatment effects were found in the periods d 83-96 and d 96-99. From d 99-106, chicks that received the optimized incubation plus hatch treatment and were subsequently reared in an enriched environment gained more weight (174.0 ± 6.9 g) than chicks in both cage reared groups (132.0 ± 7.4g for optimized-cage and 152.5 ± 7.7 g for suboptimal-cage) (incubation plus hatch treatment x rearing environment interaction, *P*=0.005). The suboptimal-enriched group was in between and did not differ from the other groups.

Plasma corticosterone levels

Plasma samples for corticosterone were collected before (d 14, d 21 and d 46 of age) and after *Eimeria* infection (d 57 and d 60 of age). No interaction between the main effects incubation plus hatch treatment and rearing environment was observed for corticosterone levels. On average there were no differences in corticosterone levels between incubation plus hatch treatments or rearing environments. However, chicks of the optimized treatment tended (P=0.08) to show higher corticosterone levels before infection (4.37 ± 0.23 ng/mL) compared with chicks of the suboptimal treatment (3.78 ± 0.20 ng/mL). This effect disappeared after *Eimeria* infection (results not shown).

IB antibody titre

No interaction was observed between the main effects incubation plus hatch treatment and rearing environment for IB antibody titre. The main effect of incubation plus hatch treatment was not significant (Figure 2), but the enriched rearing environment significantly decreased the IB antibody titre (*P*=0.009), compared to the caged rearing environment. This difference could mainly be attributed to the faster and larger increase of the IB antibody titre in cage reared chicks after IB infection at d 92.

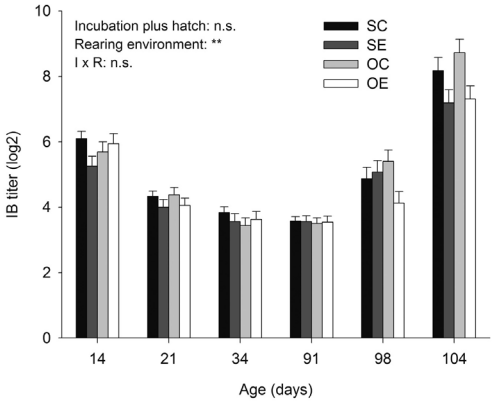

Figure 2. Effect of suboptimal (S) or optimized (O) incubation plus hatch in combination with a cage (C) or enriched (E) rearing environment from wk 0-7 of age on IB antibody titre after vaccination and infection. Chicks were vaccinated against IB at d 1 and d 15 and IB infection occurred at d 92.

Intestinal lesions

Mixed manure samples from each cage/pen did not indicate an *Eimeria* contamination before the actual infection at d 53. Dissection of the intestines at d 4 and 7 post infection (p.i.) demonstrated intestinal lesions in the duodenum *(E. acervulina)* and some in the jejunum *(E. maxima)* and caeca *(E. tenella)*. The lesions in the

jejunum and caeca were only very minimal and further analysis on these intestinal sections was not performed.

No interaction was observed between the main effects incubation plus hatch treatment and rearing environment with regard to intestinal lesions. The severity of the lesions was not affected by the incubation plus hatch treatment (P=0.68) (Figure 3a). Chicks reared in the enriched environment had less severe duodenal lesions compared with chicks reared in a cage (P=0.02). In addition, there was an effect of the day p.i. on lesion severity. At d 4 p.i. lesions were more severe than at d 7 p.i. (P<0.001).

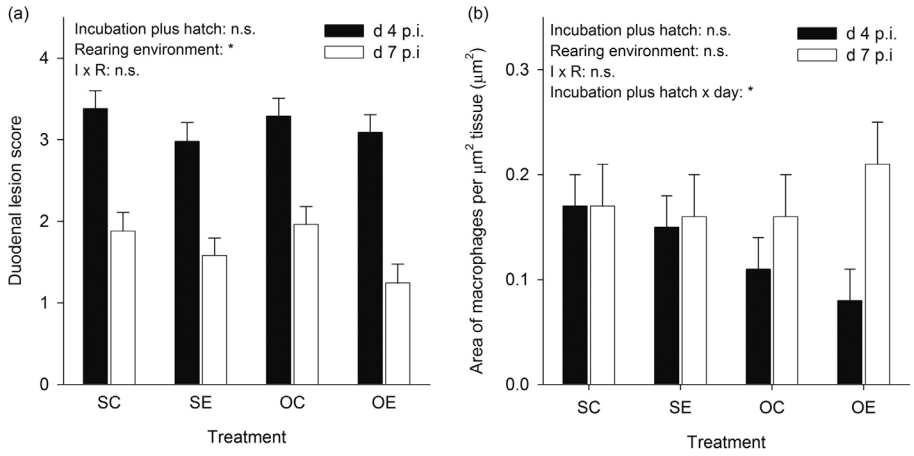

Figure 3. Effect of suboptimal (S) or optimized (O) incubation and hatch in combination with a cage (C) or enriched (E) rearing environment from wk 0-7 of age on the (a) intestinal lesions and (b) macrophage cell numbers in the duodenum, at day 4 and day 7 after a multi-species infection with sporulated oocysts of *E. acervulina* (25,000), *E. maxima* (5,600) and *E. tenella* (3,100). Intestinal lesions were scored between 0 (no lesions) and 4 (severe lesions).

Immunohistochemistry

No interaction was observed between the main effects incubation plus hatch treatment and rearing environment for the area of $CD4^+$ and $CD8^+$ T cell and macrophage populations in spleen and duodenum after *Eimeria* infection. Chicks from the suboptimal incubation plus hatch treatment had a larger area of macrophages at d 4 p.i. than optimized treated chicks (Figure 3b), whereas this difference was absent at d 7 p.i. (incubation plus hatch treatment x day p.i. interaction, P=0.03). Overall, incubation plus hatch treatment did not affect area of $CD4^+$ and $CD8^+$ T cell and macrophage populations in the spleen and duodenum (results not shown). Rearing environment did not affect area of $CD4^+$ and $CD8^+$ T cell and macrophage populations in the spleen and duodenum (results not shown).

Tonic Immobility test

No interaction was observed between incubation plus hatch treatment and rearing environment for TI duration and the number of TI inductions. Chicks from the

optimized incubation plus hatch treatment had a shorter duration of TI compared with chicks from the suboptimal treatment (87.47 ± 20.4 vs. 133.45 ± 16.9 s, respectively; *P*=0.05). Rearing environment did not affect TI duration and the number of TI induction (results not shown).

DISCUSSION

The present results demonstrated that differences in incubation plus hatch as well as early rearing environment affect the development of adaptive capacity to infectious challenges in layer chicks. Based on the knowledge from previous studies on the effect of early life experiences (Weinstock, 1997; Janczak et al. 2006), we hypothesized that an optimized incubation and hatch environment would reduce perinatal stress and result in better developed chicks, that would be able to cope with different challenges later in life. Furthermore, we hypothesized that a post natal environment that stimulates the expression of natural behaviour will also have positive effects on the development of the chicks and their adaptive capacity. The results in the current study confirm our hypotheses.

Chicks that received the optimized incubation plus hatch treatment and were subsequently reared in an enriched environment had the best performance during the *Eimeria* and IB infection. Besides the interaction, the subgroups incubation plus hatch and rearing environment also affected the ability to cope with the infectious challenges separately, however the combination of both treatments seemed to re-enforce the positive effects. Both the optimized incubation plus hatch treatment as well as the enriched rearing environment had a positive effect on BW and feed intake during the *Eimeria* infection. In addition, the optimized incubation plus hatch treatment resulted in reduced macrophage numbers in the duodenal tissue at d 4 after *Eimeria* infection, whereas the enriched rearing environment stimulated the recovery of intestinal damage caused by *Eimeria* and resulted in a decreased production of specific antibodies after IB infection.

The possible factors that caused the positive effects of optimized incubation plus hatch and enriched rearing on performance during the infections will be discussed below.

Suboptimal treated chicks were incubated at a low EST in wk 1 (36.7°C), a normal EST in wk 2 (37.8°C) and a high EST in wk 3 (38.9°C). This treatment increased embryonic mortality and lengthened incubation time compared to optimized incubation. This is in correspondence with previous studies in broilers (Lourens et al., 2005). The aim of the different incubation treatments was to create chicks differing in quality at hatch (Lourens et al., 2005). Chick quality was estimated by chick length, which tended to be longer in optimized incubated chicks, indicating a better chick quality in this group (Molenaar et al., 2008). A better chick quality is assumed to result in better later life performance, which will most likely also affect the ability of chicks to cope with (infectious) challenges.

After hatch, optimized treated chicks were transported to a more comfortable ambient temperature and had immediate and unlimited access to feed, water and foraging material. Early feed, water and foraging possibilities stimulate the natural behaviour of chicks and together with the lower ambient temperature this treatment was believed to reduce stress. Perinatal stress reduction is important, as previous studies have demonstrated that stress in early life can lead to increased emotionality, anxiety and subsequently maladaptive behaviour in later life

(Weinstock, 1997). The optimized incubation plus hatch environment in the present study resulted in less fearfulness behaviour in 47 day old chicks exposed to the Tonic Immobility test (Gallup, 1974). This indicates that the optimized incubation plus hatch treatment was indeed less stressful for the chicks. However, although perinatal stress is known to influence the capacity to adapt later in life (Weinstock, 1997), the positive effects of the optimized incubation plus hatch treatment on early growth and response to the infections are probably more related to the early provision of feed in this treatment. Optimized incubation plus hatch resulted in a lower impact of the *Eimeria* infection on performance and decreased macrophage numbers after infection in the duodenum.

Previous studies demonstrated that early feed intake (immediately after hatch) enhances the functional development of the intestines in chicks, which results in increased digestibility and thereby growth (Noy et al., 2001, Friedman et al., 2003). This explains the higher body weight of optimized chicks in the first 3 weeks post hatch in the current study. The functional development of the intestines as a digestive organ seems to be closely related to its development as a major lymphoid organ (Thompson et al., 1996). Previous studies demonstrated that early feed intake has a positive effect on the development and maturation of the gut associated lymphoid tissue (GALT), which includes the caecal tonsils, bursa of Fabricius, Peyer's patches and other lymphoid nodules and provides both local and systemic protection in young chicks (Dibner et al., 1998; Friedman et al., 2003; Kajiwara et al., 2003). Delayed access to feed has shown to decrease bursa weight (Dibner et al., 1998) and to delay the development of T and B lymphocytes in the hindgut in the first 2 weeks of life (Bar-Shira et al., 2005). Systemic and intestinal antibody responses following rectal immunization with antigen were also continuously lower in chicks with delayed access to feed (Bar-Shira et al., 2005). The possible mechanism by which early feed intake stimulates immune development in young chicks is previously discussed in the study of Dibner et al. (1998), which hypothesized that early feed intake triggers the full differentiation of the primary immune cells (particularly the B-lymphocytes) by increasing the antigen levels in the gastrointestinal tract. After differentiation of the primary immune cells, the development of secondary immune structures (e.g. GALT) is stimulated. Besides the effect of early feed intake on GALT development, some studies demonstrated that early feed intake stimulates the utilization of the yolk (Noy and Sklan, 1996; Bhanja et al., 2009). The yolk is the major source of maternal antibodies for the chick and if the utilization of the yolk is stimulated it can result in better protection against infectious agents in the first few weeks after hatch (Larsson et al., 1993). The optimized incubation plus hatch treatment did not affect the performance and immune responses of chicks during the respiratory IB infection. This suggests that the early feeding mainly contributed to better intestinal functioning and immunity, thereby reducing the effects of an intestinal pathogen, but not of a respiratory infection.

The positive effects of the enriched rearing environment on performance during the *Eimeria* and IB infection can probably mainly be attributed to the materials present in the environment. The environmental enrichment was used to create a more natural environment for the chicks, in which they were able to show and exploit more of their natural behaviours. Although behaviour was not recorded in the current study, we observed that chicks in the enriched environment showed more explorative behaviour compared to cage chicks. In addition, chicks in the enriched environ-

ment were also dust bathing frequently, which was not possible for chicks in the cage environment. Because enriched chicks seemed to be more occupied during the day, they possibly require more energy for body maintenance (Bell and Weaver, 2002), which can explain the higher feed intake (but not BW) in this group compared to the caged chicks. The enriched environment contained wood shavings and peat dust, in which numerous types of (harmless) microorganism are present, which can potentially trigger and stimulate the development of the immune system (Friedman et al., 2003). Chicks can ingest some of the microorganisms present in the peat dust and wood shavings with their beak, as part of explorative behaviour in the enriched environment. However, most contact with microorganisms in the environment occurs in the distal intestine and is mainly caused by the influx of bacteria through the cloaca. Earlier, it has been demonstrated that the presence of micro flora is associated with the development of lymphoid follicles in the gut (Honjo et al., 1993). Therefore, it is possible that the enriched rearing environment in this study has stimulated the immune system in the gut and, as a consequence, decreased the impact of an *Eimeria* infection. In addition, the immune system might also be stimulated in a broader perspective, because feed intake and BW gain after the IB infection was also higher in enriched chicks than in caged chicks. In contrast, enriched chicks had a lower IB antibody titre after infection. These results suggest that the infection in cage housed chicks was more severe than in enriched housed chicks, and more antibodies were needed to combat the infection. An increased infection severity will probably also result in less feed intake and consequently more weight loss, which can also be observed in the cage chicks.

In summary, optimized incubation plus hatch as well as enriched rearing influence the adaptive capacity of chicks to infectious challenges. When the two treatments are combined, the positive effects of both treatments are re-enforced, which can mainly be observed in a better performance of the chicks during the infections. In conclusion, the present study demonstrated that early life experiences can indeed affect the capacity of layers to cope with an *Eimeria* and IB infection at an older age. Although the contrasts in incubation plus hatch and rearing environment were not continued throughout the entire experiment, the effects remained present for weeks after the end of the contrasts. This suggests that the development of the ability to cope with infectious challenges can be influenced with management during a short period early in life and that the effect on the adaptive capacity lasts for a considerable time after cessation of the specific management.

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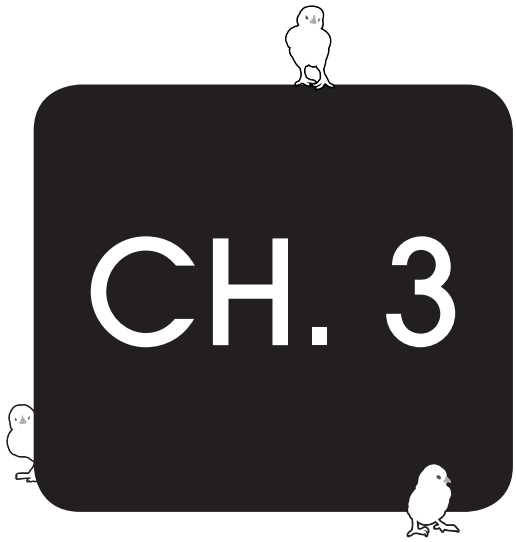

THERMAL MANIPULATION DURING LAYER CHICK EMBRYOGENESIS

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ABSTRACT
The current study investigated the effects of temperature manipulation (TM) during late embryogenesis on temperature preference, response to high environmental temperature, behaviour, and performance in young layer chicks. Control embryos (CC chicks; n=96) were incubated at 37.8°C eggshell temperature (EST) throughout incubation. Thermally manipulated embryos (n=96) were incubated at 37.8°C EST throughout incubation and exposed to 40°C for 4h/d from embryonic day (ED) 14-18 (TM chicks). After hatch, chicks from each treatment were divided into 3 subgroups (n=32 per group) and subjected to a temperature preference test at d 1, 7, or 33. One day after the temperature preference test, each subgroup was exposed to one thermal challenge for 4 h (d 2, 40°C; or d 8, 40°C; or d 34, 35°C). Effects of TM on (fearfulness) behaviour of chicks were investigated in a Tonic Immobility test and during home pen observations. TM decreased incubation time with 7 h (P<0.0001) and body temperature at hatch with 0.2°C (P=0.002). TM chicks preferred a lower ambient temperature in the temperature preference test (P<0.05) and showed a higher body temperature response than CC chicks to the thermal challenge at d 2 and d 8 (P<0.05). No effects of TM on behaviour and performance were observed.
Since most TM studies are conducted in broilers, this study is the first attempt to unravel the effects of TM during late embryogenesis on post hatch environmental adaptation in layer chicks. The results demonstrated that effects of our TM on postnatal temperature preference and response to high environmental temperatures are only found until d 8 of age. This may suggest one of three options: a. the timing, and/or the level of TM and duration were not at the sensitive period of embryogenesis and/or not sufficient, respectively; b. the level of the postnatal thermal challenge was not strong enough to induce a hyperthermic response; c. the postnatal effects of TM in layers are limited in time.

Keywords: thermal manipulation; laying hen; adaptation; thermal challenge; temperature preference;

INTRODUCTION

Incubation temperature can have a significant effect on hatchability, chick quality and later life performance in poultry and is considered to be the most important environmental factor during egg incubation (Decuypere and Michels, 1992; Lourens et al., 2005). Barott (1937) was one of the first to investigate the importance of incubation temperature and demonstrated that an incubation temperature of 37.8°C resulted in highest hatchability and chick quality in layers. According to Barott, deviations from this optimum should not be more than ± 0.3°C but it was not demonstrated what the effect of high or low incubation temperatures in certain developmental periods of the embryo could be. During embryogenesis, the development and maturation of functional systems is divided over different periods. Some are maturing early, others late (Decuypere and Michels, 1992). During certain 'critical periods' in embryonic development, the environment experienced by the embryo can pre-determine the actual set-point of these functional systems for the entire post hatch life. This can result in epigenetic adaptation (Dorner, 1974, Nichelmann et al., 1999, Tzschentke and Plageman, 2006). Epigenetic adaptation is the adaptation of an animal to an expected environment (Nichelmann, 1992) Incubation temperature is one of the environmental factors that can induce epigenetic adaptation of different physiological control systems.

High or low incubation temperatures at the end of embryonic development can induce epigenetic temperature adaptation (Minne and Decuypere, 1984; Tzschentke et al., 2004). Epigenetic temperature adaptation is suggested to be the result of set-point changes in the immature thermoregulatory and feedback mechanisms of the embryo (Arjona et al., 1988), which results in alterations in the thermoregulatory threshold response (Yahav, 2009) and increased adaptation to postnatal hot or cold environments. For instance, chicks exposed to high incubation temperatures during late embryogenesis can adapt better to high environmental temperatures post hatch (Tzschentke, 2007, 2008).

Besides the thermoregulatory system, high incubation temperatures during late embryogenesis might also affect another important control system, the hypothalamic–pituitary–adrenal (HPA) axis (Debonne et al., 2008). From ED 14 onwards, functional development and maturation of the HPA-axis occurs (Jenkins and Porter, 2004). When the HPA-axis is fully functional, the secreted adrenocortical steroids have a significant influence on the metabolism of the post hatch chick, but also on its development, stress tolerance, and numerous other functions that are important and critical for its growth, post hatch survival, and performance (Jenkins and Porter, 2004). Increasing incubation temperatures in the period of functional development of the HPA-axis are likely to affect the set-point of this control system, resulting in long lasting effects on different body functions and behaviour. In summary, high incubation temperatures can affect the post hatch development of chicks, dependent on both the period and duration of the application (Decuypere and Michels, 1992; French, 2000).

Increasing the incubation temperature during certain developmental periods might also be beneficial for the poultry industry. In practice, standard egg incubation profiles are based on maintaining a constant temperature throughout incubation. As the embryo grows and produces more metabolic heat, the incubation temperature is decreased somewhat to prevent overheating of the embryo, but large changes in incubation temperature do not occur. These constant incubation temperature

profiles are mainly aimed at achieving maximum hatchability, but little is known about the consequences of these incubation profiles for chick performance and development in later life. Instead of maintaining a constant temperature throughout incubation, increasing the temperature during certain periods of embryonic development might stimulate the development of different physiological control systems and body functions of the embryo and might thereby increase the adaptation capacity of chicks. Increasing the adaptation capacity of chicks can be beneficial for their performance in different environmental circumstances in later life (Tzschentke and Halle, 2009).

Previous studies on the effects of manipulation with high temperatures during embryogenesis have primarily been conducted in broiler chickens (Yahav et al. 2004a,b; Piestun et al. 2008, 2009) and Muscovy ducklings (Nichelmann, 2004), but the effects in rearing hens have never been investigated. Therefore, we hypothesized that temperature manipulation with high temperatures during certain periods in late incubation in layers could induce epigenetic (temperature) adaptation and affect the adaptation capacity and performance of the chicks post hatch. The current study was performed to investigate this hypothesis, by using a temperature manipulation of 40°C for 4 h from ED 14-18. Post hatch effects on performance, behaviour, temperature preference, and response to high environmental temperatures were investigated.

MATERIALS AND METHODS

All procedures in this study were approved by the Animal Care and Use committee of Wageningen University in the Netherlands.

Incubation Treatment, Embryo Mortality and Hatchling Measurements

Two hundred and ninety eggs of a Lohmann brown layer breeder flock with an age of 44 weeks (Verbeek Hatchery, Lunteren, the Netherlands) were weighed and randomly divided over two treatments. Eggs were incubated at a constant EST of 37.8°C and 55-60% RH throughout incubation (CC treatment) or at an EST of 37.8°C and 55-60% RH throughout incubation but treated for 4 h per day at 40°C from ED 14-18 (TM treatment). The 2.2°C EST increase from 37.8°C until 40°C took approximately 10 minutes and the 4 h period was calculated from the start of the increase. Incubation occurred in climate respiration chambers and one chamber was used per treatment (Lourens et al., 2006). Eggs in both treatments were turned each hour. At ED 7, infertile eggs or eggs containing dead embryos were removed from the incubator after candling and visually examined to determine fertility or moment of mortality (Lourens et al., 2006). At ED 19, eggs of both treatments were placed in individual hatching baskets. The machine temperature applied to obtain an EST of 37.8°C at ED 19 was maintained for the remaining incubation period.

During ED 19, 20 and 21, eggs were checked for hatching every three hours. Nine hour old chicks were collected and BW and chick length (Hill, 2001; Molenaar et al., 2008) were measured and cloaca temperature (T_b) was recorded with a digital thermometer (measurement accuracy ±0.1°C, MT1831, Microlife®, Widnau, Switzerland). After completion of the measurements, chicks remained in the incubator until all eggs had hatched. After the hatching period, the un-hatched eggs were removed from the incubator and also visually examined to determine moment of embryonic mortality (Lourens et al., 2006).

Postnatal Conditions, Temperature Preference and Thermal Challenge

After hatch, 96 chicks from each treatment were divided into 3 subgroups of 32 chicks per treatment. Groups of 4 chicks of the same incubation treatment were housed in climate respiration chambers (Verstegen et al., 1987) in pens containing wood shavings and a perch. Pens were located in a temperature controlled room (T_a= 33°C at d 1 linearly decreasing until 23°C at d 36) with 23 h light (darkness from 00:00-01:00). Water and commercial available feed (202 g/kg crude protein, 11.1 MJ of ME/kg) were provided *ad libitum*.

Each subgroup, consisting of 32 CC and 32 TS chicks, was subjected to one temperature preference test and one thermal challenge. The temperature preference tests were performed at d 1 (subgroup 1), or d 7 (subgroup 2) or d 33 (subgroup 3), followed by a thermal challenge at the subsequent day, thus d 2 (subgroup 1), d 8 (subgroup 2) or d 34 (subgroup 3). The chicks of subgroup 1 and 2 were removed from the experiment after the thermal challenge (d 2 and d 8 respectively), whereas chicks from subgroup 3 were reared until the end of the experiment (d 36). The division into subgroups was necessary to prevent potential thermal conditioning with high temperatures in the transition phase of the chicks, which is approximately until d 10 post hatch (Tazawa et al., 1988; Nichelmann and Tzschentke, 2002).

Temperature preference of the chicks was measured during a test, based on the method of Myhre et al. (1975). A wooden box (160 x 60 x 50 cm) with a Plexiglas lid and wood shavings on the bottom, contained 24 temperature sensors divided over the floor area. Two infrared lights (250 Watt) were placed on one side of the box, creating a temperature gradient from 20°C until 50°C over the entire length of the box. T_a in the box was recorded by all 24 sensors each minute and send to a computer database. Video cameras were placed above the box to record every test. Four chicks from the same pen were placed in the middle of the box and observed for 30 min. The location of the chicks was written down at the end of each test and the ambient temperature of each location could be calculated with the temperature sensor data. T_b was measured before and after the temperature preference test.

For the thermal challenge, chicks were transported to another climate respiration chamber where they were housed in the same social group of 4 chicks per pen. The ambient temperature was increased for 4 h to 40°C at d 2 and 8 and to 35°C at d 34. The ambient temperature was lower at d 34 to prevent mortality at this age. RH was maintained at 55-65% during all thermal challenges. T_b was measured before, right after and 30 minutes after the thermal challenge.

During the experiment, BW and feed intake of chicks in subgroup 3 was measured weekly.

BEHAVIOUR

Tonic Immobility Test

Two individuals from each pen in subgroup 3 of the TS and CC treatment were subjected to a Tonic Immobility (TI) test (Jones et al., 1994) at d 14 post hatch, to measure fearfulness behaviour. Chicks were restrained for 15 sec to induce TI. When induction was not successful, the procedure was repeated until a maximum of four restraints per individual. Unsuccessful induction of TI resulted in a score of 0 sec. When induction was successful, the duration was recorded for a maximum of 600 sec. The recording ended after the chicken righted itself, or when the time limit was reached. In the latter situation, the score given was 600 sec.

Home Pen Behaviour

To measure behavioural differences in the home pen environment, behaviour in subgroup 3 was observed at one day in wk 1, 2 and 3 using a 2-min scan sampling method from 9.00 until 12.00 am. With this method, each chick's behavioural state (see Table 1) was recorded every 2 minutes for 3 hours long. Thus, within a sampling interval of 2 minutes every chick (64 in total, 4 per cage) was scored once. This resulted in a total of 90 behavioural state scores per chick per day. After the observation, the percentage of time spent on a certain behaviour was calculated from the data.

Table 1. Ethogram for home pen observations in week 1, 2 and 3

Behaviour	Description
Inactive	Sitting or standing with eyes open or closed without performing any other type of behaviour
Explore	Pecking floor/pen, scratching floor/pen
Play	Chasing, play fighting
Ingestive	Ingesting feed from the feed tray or water from the water nipples
Dust bathing	Forcing wood shavings into the plumage by squatting on the ground and making appropriate movements with the body, wings and legs
Comfort behaviour	Preening, wing flapping, stretching, feather ruffling
Walking	Walking without performing any other type of behaviour
Pecking	Gently pecking the feathers of a pen mate

Statistical Analysis

For statistical analysis we used SAS software (SAS Institute Inc., Cary, NC, USA). Differences in hatchability, embryonic mortality and number of TI inductions were analysed with the Chi-square test for the effect of EST treatment. Incubation time, BW at hatch, T_b at hatch, chick length at hatch, TI duration and temperature preference were analysed with a PROC GLM with EST treatment as fixed effect. Differences in Tb before and after the temperature preference test and thermal challenge were analysed with a PROC MIXED for repeated measurements with EST treatment, time and their interaction as fixed effects. A PROC MIXED for repeated measurements was used for BW and feed intake to determine effects of EST treatment, time and their interaction. T_b before and after thermal challenge, tonic immobility duration an overall feed intake data were transformed with a log10 transformation to obtain normal distributed data. Data are presented as means ± standard errors in Tables and Figures. Behavioural data from home pen observations were transformed with an arcsine transformation and analysed with a PROC MIXED for the effects of EST treatment, time and their interaction. Non-significant interactions were excluded

from analysis by stepwise deletion. Effects were considered significant at $P<0.05$.

RESULTS

Hatch

Temperature manipulation during ED 14-18 did not affect embryonic mortality, hatchability, BW at hatch, and chick length (Table 2), but decreased incubation time with approximately 7 h ($P<0.001$).

Average body temperature of TM chicks at hatch was 0.2°C lower compared to CC chicks ($P=0.002$). Hatched chicks were divided in early (469-478h), mid (481-487h) and late (490-498h) hatchers. Late hatchers had a significant higher body temperature (40.1°C) compared with early and mid-hatchers (39.2°C), independent of their incubation treatment ($P<0.001$). In addition, there was a higher percentage of early hatchers in the TM treatment (36.5%) compared with the CC treatment (8.3%), whereas there was a higher percentage of late hatchers in the CC treatment (46.8%) compared with the TM treatment (8.3%) .

Table 2. Embryonic mortality, hatchability, incubation time, body temperature (T_b) and chick quality of control incubated chicks (CC) and chicks exposed to temperature manipulation during late incubation (TM)

	Treatment[1]			
Item	CC	TM	Pooled s.e.m.	P-value
Embryonic mortality, %				
wk 1	3.82	6.67	-	n.s.
wk 2	3.82	2.67	-	n.s.
wk 3	3.82	8.00	-	n.s.
Hatch of fertile, %	88.54	82.67	-	n.s.
Incubation time, h	487.5	480.2	0.42	***
BW, g	42.8	42.4	0.22	n.s.
Chick length, cm	17.5	17.4	0.04	n.s.
T_b at hatch, °C	39.5	39.3	0.04	**

[1]Treatments: CC = control (constant EST of 37.8°C in wk 1, 2 and 3); TM: temperature manipulation (constant EST of 37.8°C in wk 1, 2 and 3 + 4 h treatment at 40°C at ED 14-18).

*$P<0.05$; **$P<0.01$; ***$P<0.001$; n.s.= not significant

Temperature Preference Test

The temperature preference test was performed at d 1, 7 and 33 post hatch. Only data of d 1 and d 7 was used in the analysis, because the preference box was too small for reliable measurements at d 33. TM chicks had a lower preferred ambient temperature at d 1 (P=0.002) and d 7 (P=0.03) post hatch, compared to CC chicks (Table 3). At d 1 and d 7 in both treatments, T_b was lower before the temperature preference test compared to after the test (P<0.001). In addition, no differences in T_b before and after the test were found between incubation treatments.

Table 3. Body temperature (Tb) before and after the temperature preference test and the preferred ambient temperature (Ta pref) of one- and seven-day-old control incubated (CC) and chicks exposed to temperature manipulation during late incubation (TM)

			Treatment[1]		P-value		
	Day	Timing	CC	TM	Pooled s.e.m.	Treatment	Time
T_b, °C	1	before	40.8	40.5	0.02	n.s.	***
	1	after	41.5	41.5			
	7	before	40.9	40.9	0.02	n.s.	***
	7	after	41.6	41.7			
T_a pref, °C	1		30.9	29.9	0.25	**	-
	7		28.1	27.7	0.16	*	-

[1]Treatments: CC = control (constant EST of 37.8°C in wk 1, 2 and 3); TM: temperature manipulation (constant EST of 37.8°C in wk 1, 2 and 3 + 4 h treatment at 40°C at ED 14-18).

*P<0.05; **P<0.01; ***P<0.001; n.s.= not significant

Thermal Challenge

After 4 h of thermal challenge, the TM chicks had a higher Tb than the CC chicks at d 2 (P=0.03) and d 8 (P=0.05), but not on d 34 (P=0.44) (Figure 1). At d 2, the difference in Tb between treatments was still present 30 minutes after the thermal challenge (P=0.001), whereas this difference was absent at d 8 and 34. Tb before the thermal challenge at d 2, 8, and 34 did not differ between the TM and CC chicks.

BW and Feed Intake

No differences in BW and feed intake were observed between TM and CC chicks throughout the experiment (results not shown).

Behaviour

Tonic Immobility Test

Neither TI duration (P = 0.90) nor the number of TI inductions (P=0.75) were affected by incubation treatment. TM chicks had an average TI duration of 142.0 ± 41.18 sec (1.25 ± 0.11 inductions) compared with 121.69 ± 23.88 sec (1.31 ± 0.15 inductions) in CC chicks.

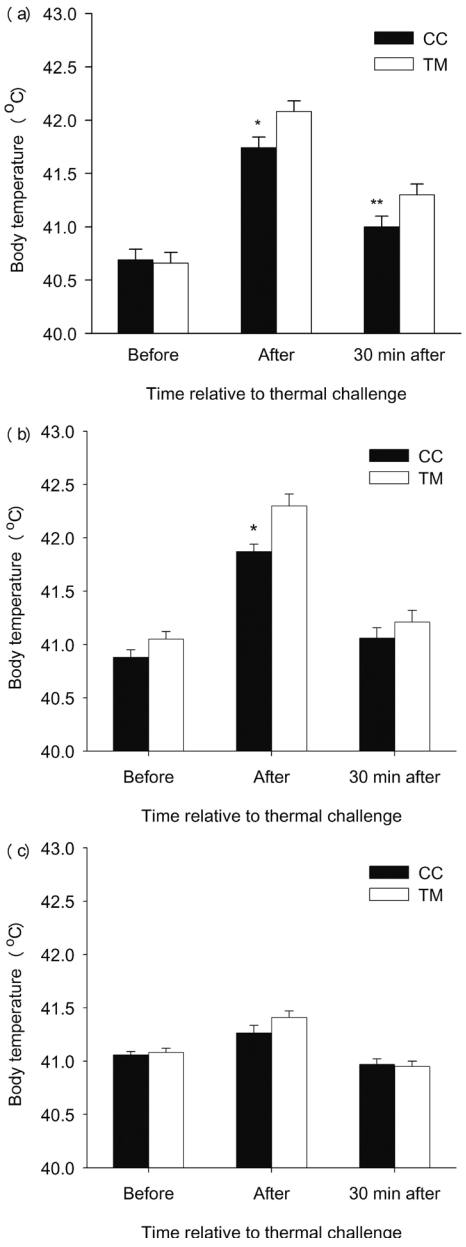

Figure 1. Effect of temperature manipulation during late embryogenesis on body temperature before, immediately after and 30 min. after a 4 h thermal challenge at d 2 (A), d 8 (B) and d 34 (C) post hatch. Ambient temperature during the thermal challenge was 40°C at d 2 and d 8 and 35°C at d 34. CC represents the control treatment with a constant EST of 37.8°C in wk 1, 2 and 3 of incubation. TM represents the temperature manipulation treatment with a constant EST of 37.8°C in wk 1, 2 and 3 of incubation + 4 h treatment at 40°C at embryonic d 14-18. Both * (P<0.05) and ** (P<0.01) represent differences between incubation treatments.

Home Pen Behaviour

No significant differences in behaviour between CC and TM chicks were observed during the home pen observations (Table 4).

Table 4. Percentage (mean) of observations spent on behaviour types during a 2 min-scan sampling interval from 9:00-12:00 am in week 1, 2 and 3 of control incubated chicks (CC) and chicks exposed to temperature manipulation during late incubation (TM)

	Treatment[1]						P-value		
	Week 1		Week 2		Week 3		Treatment	Week	Treatment*Week
Behaviour, %	CC	TS	CC	TS	CC	TS			
Play	0.03	0.3	0.5	0.5	1.0	1.1	n.s.	***	n.s.
Explore	17.6	20.7	23.5	21.9	16.0	22.8	n.s.	n.s.	n.s.
Pecking	0.5	0.4	0.0	0.2	0.4	0.4	n.s.	*	n.s.
Inactive	54.5	50.8	47.5	49.3	56.7	50.8	n.s.	n.s.	n.s.
Comfort	7.0	7.4	12.4	11.6	9.0	8.7	n.s.	**	n.s.
Ingestive	12.3	12.6	11.6	12.5	10.2	11.1	n.s.	n.s.	n.s.
Dust bathing	1.6	1.3	0.5	0.6	2.7	1.6	n.s.	**	n.s.
Walking	4.8	5.1	4.0	3.4	3.6	3.5	n.s.	*	n.s.
Other	1.7	1.4	0.0	0.0	0.4	0.0	-	-	-

[1]Treatments: CC = control (constant EST of 37.8°C in wk 1, 2 and 3); TM: temperature manipulation (constant EST of 37.8°C in wk 1, 2 and 3 + 4 h treatment at 40°C at ED 14-18).

*P<0.05; **P<0.01; ***P<0.001; n.s.= not significant

DISCUSSION

The objective of the current study was to investigate effects of TM during late embryogenesis on temperature preference, response to high environmental temperatures, performance, and behaviour in young layer chicks. TM during late embryogenesis reduced incubation time of the embryos, which is in correspondence with previous studies on TM during incubation (Decuypere, 1984; Janke et al., 2002; Yahav et al., 2004a,b). In addition, the TM treatment resulted in chicks with a 0.2°C lower T_b at hatch. This difference in T_b can probably be explained by the hatch distribution. More early hatchers with a lower T_b were observed in the TM treatment, whereas more late hatchers with a higher T_b were observed in the CC group.

There were no indications that the TM treatment had negative effects on survival and viability of embryos, because embryonic mortality and hatchability did not differ between incubation treatments. Other studies on TM during embryogenesis found conflicting results on hatchability, which might be related to differences in timing, load and duration of the manipulations (Yahav, 2004a,b; Collin, 2005). In our study, BW at hatch and chick length was not affected by the temperature treatment, which indicates that chick quality did not differ between CC and TM chicks.

TM in the current study decreased the preferred ambient temperature of chicks

compared to CC chicks at d 1 (subgroup 1) and d 7 (subgroup 2) post hatch. This suggests that TM has affected the thermoregulatory system in a positive manner, because previous studies have shown that it is beneficial to prefer lower temperatures under non challenging conditions, as part of a thermal strategy to save energy (Tzschentke, 2007). Interestingly, the T_b of both CC and TM chicks was lower before the temperature preference test than after the test, whereas the chicks preferred an ambient temperature that was lower than their housing temperature. The reason for this increase in T_b is unknown, however, it might be stress related, because chicks were handled two times before the test, their T_b was measured and they were placed in a new environment.

When the chicks were exposed to a thermal challenge of 40°C (4 h) at d 2 (subgroup 1) and d 8 (subgroup 2), TM chicks had a higher T_b response compared to CC chicks. In contrast, the thermal challenge of 35°C for 4 h at d 34 of age, did not result in any differences in T_b response between TM and CC chicks. The differences in response to the thermal challenge between TM and CC chicks until d 8 of age also indicate that the thermoregulatory system has been affected by the TM applied. This confirms our hypothesis. However, in contrast to the chicks' responses in the current study, Tzschentke (2007) demonstrated that embryos exposed to high temperatures (38.5°C) during late incubation had a preference for higher ambient temperatures and the study of Yahav et al. (2004b) demonstrated a lower T_b response to thermal challenge in chicks exposed to TM during late embryogenesis.

The major difference between these studies and our study is that we did not use a meat producing breed, but a layer breed. Laying hens are probably more efficient in coping with heat stress and loosing heat due to their smaller body size, lower metabolism and growth compared to meat producing breeds at the same age. It is therefore possible that the thermoregulatory adaptive response was not challenged in this study, because the temperature during the thermal challenge was not high enough to induce a strong hyperthermia. When investigating thermal tolerance, a strong hyperthermia is needed to demonstrate any differences between the treatments applied. In the current study, the T_b of thermally challenged chicks increased with only 0.3°C at d 34, which cannot be referred to as a hyperthermic response.

Previous studies have demonstrated that TM during embryonic development can affect postnatal neuronal thermosensitivity (Piestun et al., 2008) by changing the thermoregulatory threshold response (Yahav, 2009). The differences in temperature preference between TM and CC chicks do suggest that TM has affected the thermoregulatory system, however to support this hypothesis we would also need more information on physiological parameters (T_3, T_4, and corticosterone) during the temperature preference test (and thermal challenge).

Furthermore, we hypothesized that TM in the current study might affect the behaviour and later life performance of the chicks, because it was applied during a sensitive period of HPA-axis development and maturation (Jenkins and Porter, 2004). However, no differences in BW, feed intake and behaviour were found. It is possible that the timing or duration TM in this study was not optimal to change any future responses related to the functioning of the HPA-axis. Increasing the incubation temperature for a longer period or perhaps adjusting the timing of the manipulation in future experiments could give some more information.

To our knowledge, there are no studies available of TM with high temperatures during embryogenesis in layers. Therefore, the current study is the first attempt to un-

ravel the effects of TM with high temperatures during late incubation on post hatch environmental adaptation in layer chicks. Effects of our TM on postnatal temperature preference and response to high environmental temperatures are only found until d 8 of age. This may suggest one of three options: a. the timing, and/or the level of TM and duration were not at the sensitive period of embryogenesis and/or sufficient, respectively; b. the level of the thermal challenge was not strong enough to induce a hyperthermic response; c. the postnatal effects of TM are limited in time.

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ADAPTIVE RESPONSE TO EIMERIA ACERVULINA IN REARING HENS IS AFFECTED BY SUBOPTIMAL INCUBATION AND HEAT EXPOSURE

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ABSTRACT

The current study aimed to investigate whether suboptimal temperature in wk 1 and wk 3 of layer embryo incubation affects their development and post hatch adaptive capacity during infectious challenges, by using *Eimeria* as a model infection under normal and immediately after more challenging environmental conditions of 72 h heat exposure. Eggs (n=160 per treatment) were incubated at optimal (**OI**=37.8°C continuously) or suboptimal (**SI**) eggshell temperature (EST; 36.7°C-37.8°C-38.9°C in wk 1, 2, and 3, respectively). At d 33 of age, half the chicks of each incubation treatment were exposed to 72 h heat (35°C), whereas the other half remained under control conditions (21°C). At d 36 of age, all chicks were inoculated with 1 ml of a PBS solution containing 25.000 sporulated *E. acervulina* oocyst/mL. The adaptive response to *E. acervulina* was measured by BW gain and feed intake from d 0-3 p.i., d 3-5 p.i and d 5-7 p.i, and by oocyst production (d 4 and 7 p.i) and lesion scores in the duodenum (d 3, 4 and 7 p.i.).

Our results demonstrated that suboptimal temperatures in wk 1 and wk 3 of incubation resulted in a reduction in yolk free body weight, chick length and navel condition. Moreover suboptimal incubation temperature tended to reduce the adaptive capacity to *Eimeria acervulina*. This was demonstrated by tendencies to lower feed intake and BW gain, more duodenal lesions and higher oocyst production after inoculation of *E. acervulina*. Higher lesion scores and faecal oocyst numbers were especially found when suboptimal incubation was combined with heat exposure preceding the infection.

In conclusion, suboptimal incubated layer chicks tend to be less able to cope with an infectious challenge post hatch. This study therefore emphasizes the importance of incubation temperature for layer embryos in the development of their ability to cope with post hatch challenges.

Keywords: rearing hen; incubation temperature; adaptive capacity; heat exposure

INTRODUCTION

In practice, layer hens encounter different environmental challenges which may affect their health and welfare. In order to respond to these challenges with the appropriate behavioural, physiological and immunological adjustments, a well-developed adaptive capacity is necessary. Besides genetic background, environmental conditions and experiences in early pre- and postnatal life are known to influence the development of adaptive capacity (Star, 2008; Walstra et al., 2010).

For layer hens, development starts in ovo where embryos can already be influenced by environmental conditions. Temperature is one of the most important environmental factors for development of the chicken embryo. Previous studies in broilers and layers demonstrated that deviations from optimum incubation temperature (between 37°C and 38°C; Wilson, 1991) have negative effects on embryo development (Romanoff, 1972; Shafey, 2004; Molenaar et al. 2010a,b), hatchability (Deeming and Ferguson, 1991; Decuypere and Michels, 1992), chick quality (Lourens et al., 2005; Molenaar et al. 2010a,b) and subsequent performance (Decuypere, 1979; Lourens et al., 2005). Walstra et al. (2010) previously demonstrated that the combination of suboptimal incubation, hatch and rearing management reduces the capacity of layer hens to adapt to infectious challenges as compared to layers hatched and reared under more optimized conditions. Whether incubation temperature as a single early life condition can also affect the adaptive capacity of layers during infectious challenges has, to our knowledge, never been investigated. Because incubation temperature can easily be manipulated in practice, it could be a useful tool to create layers with a better adaptive capacity. Ultimately this will benefit their production, health and welfare.

Therefore, the objective of the present study was to investigate the effects of suboptimal incubation temperature on the development and behaviour of layer hens post hatch, and on their adaptive capacity during infectious challenges. In order to challenge the adaptive capacity, we used an infection model of E. acervulina under normal and following a presumed stressful period of heat exposure. Based on previous studies described above, we expected that suboptimal incubation temperature would reduce (embryonic) development and hypothesized that reduced (embryonic) development due to suboptimal incubation temperature will also result in a lower capacity to adapt to post hatch challenges.

MATERIALS AND METHODS

All experimental protocols were approved by the Animal Use and Care Committee of the Wageningen University, the Netherlands.

Incubation

Eggs (n = 800, Lohmann Brown; breeder flock age 42 wk, Verbeek Hatchery, Lunteren, the Netherlands) were randomly divided over two eggshell temperature (EST) treatments during incubation. Eggs were incubated at a considered optimum EST of 37.8°C in the optimized incubation (**OI**) treatment (Lourens et al., 2005). In the suboptimal incubation (**SI**) treatment, eggs were incubated at an EST of 36.7°C from embryonic day (**ED**) 0-7, 37.8°C from ED 8-14 and 38.9°C from ED 15-21. The SI treatment was based on conditions that occur in practice, where a relatively constant air temperature (± 37.8°C) is often used as treatment applied to the eggs. However, due to the imbalance between embryonic heat production and heat transfer in

early and late incubation, the embryo temperature during early incubation is lower than the incubator temperature. On the other hand, a higher embryo temperature compared to incubator temperature is observed at the end of incubation, when the embryo is producing heat itself (French, 1997; Hulet et al. 2007). The aim of the SI treatment was to mimic embryo temperature during incubation in commercial hatcheries (especially multi-stage incubators) and was also based on a previous study of Walstra et al. (2010)

Incubation occurred in climate respiration chambers (Verstegen et al., 1987) where the EST was recorded continuously and chamber temperatures were adjusted to maintain the set EST for both treatments. Relative humidity was maintained at 55% in both EST treatments. At ED 19, all eggs were candled and infertile eggs or eggs containing dead embryos were removed and visually examined to determine the moment of embryonic mortality. The fertile eggs were placed in hatching baskets. The chamber temperature applied to obtain the EST of ED 19 was maintained at ED 20 and 21. After the hatching process, un-hatched eggs were visually examined to determine moment of embryonic mortality.

Hatch

At ED 20 and ED 21, eggs were checked every 3 hours to examine the number of chicks that emerged from the eggshell and dry chicks were colour sexed. To investigate whether suboptimal incubation temperature created differences in embryonic development and quality of hatched chicks, several measurements were performed. Nine hour old female chicks were weighed, chick length (Hill, 2001; Molenaar et al., 2008) was measured and cloaca temperature (T_b) was recorded with a digital thermometer (MT1831, Microlife®, Widnau, Switzerland). Navel condition was scored as 1 (closed and clean navel area), 2 (black button up to 2 mm or black string), or 3 (black button exceeding 2 mm or open navel area) (Molenaar et al., 2010). After measurements, female chicks of both EST treatments were kept in the different hatching baskets in the same climate respiration chamber with access to feed (commercially available feed, ad libitum; 175 g/kg crude protein, 11.31 MJ ME/kg, ileal digestible lysine: 0.37%), water and wood shavings until all eggs hatched.

Nine hour old male chicks (n=20 per EST treatment) were weighed, chick length was measured, and navel condition was scored. After the measurements, chicks were decapitated and blood was collected in heparinized tubes. Blood samples were centrifuged at 3,000 rpm for 15 min. and plasma was stored at -20°C for further analysis of thyroxin (T_3) and triiodothyronine (T_4) levels by radioimmunoassay (Darras et al., 1992). T_3 and T_4 levels were measured to determine possible metabolic differences between SI and OI chicks at hatch. After blood sampling, yolk free body mass (YFBM), residual yolk (RY) weight and heart weight were determined. The remaining male chicks were not used for measurements and euthanized by CO_2.

Housing conditions

After hatch, hen chicks (n = 160 per EST treatment) were housed in separate floor pens (350 x 150 x 100 cm; one per treatment) with wood shavings in a temperature controlled room from d 1 (defined as the day after 21 d of incubation) until d 7 of age. The ambient temperature was 33°C at d 1 and decreased linearly until 31°C at d 7. All chicks had unlimited access to the same feed as used before (commercially available feed, *ad libitum*; 175 g/kg crude protein, 11.31 MJ ME/kg,

ileal digestible lysine: 0.37 %), and water.
At d 8 of age, chicks were transported to a new rearing environment, where they were divided over 32 floor pens (100 x 75 cm) containing wood shavings and a perch. Ten chicks per EST treatment were housed per floor pen and feed and water were provided *ad libitum*. The floor pens were located in two temperature controlled rooms, with 8 pens per incubation treatment (16 pens in total) per room. Ambient temperature in the rooms was 31°C at d 8 and decreased linearly until 20°C at d 38 until d 61. The light dark cycle was 24:0 from d 1-3, 18:6 LD from d 4-7, 16:8 LD from d 8-13, 14:10 LD from d 14-20, 12:12 LD from d 21-27, 11:13 LD from d 28-34, 10:14 LD from d 35-41 and 9:15 LD from d 42 until d 61. Body weight and feed intake was measured to determine growth differences between EST treatments. Chicks were weighed at d 1, 7, 11, 19, 26, 29, 36, 39, 40, 41, 42, 43, 49, 56, and 61. Feed trays were weighed empty and with feed before arrival of the chicks and subsequently at d 7, 11, 19, 26, 39, 41, 43, 49, 56 and 61 to determine feed intake.

Temperature preference of chickens
The temperature preference test was performed at d 1 and d 7 of age to investigate whether periods of suboptimal incubation temperature act on the immature thermoregulatory system of the embryo. Temperature preference of the chicks was measured during a test, based on the method of Myhre et al. (1975). A wooden box (160 x 60 x 50 cm) with a Plexiglas lid and wood shavings on the bottom, contained 24 temperature sensors divided over the floor area. Two infrared lights (250 Watt) were placed on one side of the box, creating a temperature gradient from 20°C until 50°C over the entire length of the box. T_a in the box was recorded by all 24 sensors each minute and send to a computer database. Video cameras were placed above the box to record every test. Three chicks from the same pen were randomly selected, placed in the middle of the box and observed for 30 min. The location of the chicks was written down at the end of each test and the ambient temperature of each location could be calculated with the temperature sensor data. T_b was measured before and after the temperature preference test.

Tonic Immobility Test
At d 15 of age, two chicks per pen were subjected to a Tonic Immobility (TI) test (Jones et al., 1994) to investigate whether periods of suboptimal incubation temperature act on the immature HPA-axis and thereby result in differences in fearfulness behaviour. To induce TI, chicks were placed on their back and restrained with one hand over the sternum for 10 sec. If TI could not be induced in the first restraint, the procedure was repeated until a maximum of 4 restraints. If TI could not be induced after 4 restraints, the score was 0 sec. When induction was successful, the duration was recorded for a maximum of 300 sec. The maximum time was based on the previous study by Walstra et al. (2010), which showed that most chicks tested did not reach the a TI duration of 300 sec. The recording ended after the chicken righted itself, or when the time limit was reached. In the latter situation, the score given was 300 sec. Chicks with a longer TI duration are considered to be more fearful (Jones et al., 1994).

Manual Restraint Test

At d 25 of age, 2 chicks per pen, which were not used for the TI test, were subjected to a manual restraint to investigate whether periods of suboptimal incubation temperature act on the immature HPA-axis and thereby result in differences in stress response behaviour and endocrinology. Immediately after the chick was removed from the pen, a blood sample (1 mL) was taken from the wing vein and collected in a heparinized tube. Following this procedure, chicks were put on their side and restrained with one hand for 5 minutes. Latency time to vocalize and struggle, as well as number of vocalizations and struggles during the restraint were scored. Immediately after the 5 min. restraint, a blood sample (1 mL) was taken from the other wing vein. Blood samples were centrifuged at 3,000 rpm for 15 min and plasma samples were stored at -20°C for further analysis of corticosterone levels as described by Lin et al. (2008).

Heat exposure

At the age of 33 d, the ambient temperature in one room was increased to 35°C (RH 55%) for 72 h, thereby exposing 8 floor pens per EST treatment to a high ambient temperature until d 36 of age. The other 8 floor pens per EST treatment remained at the normal (control) temperature (± 21°C). T_b of 2 chicks per pen was measured with a digital thermometer before and after 36 h of heat exposure (MT1831, Microlife®, Widnau, Switzerland). Approximately 1 h before and immediately after 72 h of heat exposure, blood samples were taken from the wing vein of 2 chicks per pen (different chicks than for TI and manual restraint test) and collected in heparinized tubes. Plasma was obtained by centrifugation at 3,000 rpm for 15 minutes and samples were stored at -20°C for further analysis of T_3, T_4 (Darras et al., 1992), and corticosterone (Lin et al., 2008).

Eimeria acervulina challenge

At d 36 of age, all chicks were inoculated in the crop with a 1 mL PBS solution containing 25,000 sporulated oocysts of *Eimeria acervulina* at d 36 of age (Animal Health Service, Deventer, the Netherlands). At d 3, 4, and 7 post-infection (p.i.), one chick from each pen was dissected and the duodenum was visually scored for lesions. The severity of the lesions ranged from 0 until 4 in which score 0 = no lesions; score 1 = 1 to maximum 5 lesions per cm^2; score 2 = more than 5 separate lesions per cm^2; score 3 = some lesions are merged but separate lesions are still visible; score 4 = lesions are merged and hardly visible individually (Animal Health Service, Deventer, the Netherlands). A faecal sample (± 2.0 g) was also collected from the dissected chicks and the number of *E. acervulina* oocysts secreted per gram faeces was counted with the salt floatation method described by Long and Rowell (1975). Both lesions and oocyst production are a measure of the severity of an *Eimeria* infection.

Statistical analysis

For statistical analysis we used SAS software (SAS Institute Inc., Cary, NC, USA). Differences in hatchability, navel condition, duodenal lesion score, and number of TI inductions were analysed with the Chi-square test for the effect of EST treatment. For navel condition, the person scoring was added as a class variable. Chick was the experimental unit. Incubation time, BW at hatch, chick length at hatch, T_b at hatch, heart weight, YFBM, RY weight, TI duration, struggles and vocalizations during

manual restraint, and temperature preference were analysed with a PROC GLM for the effects of EST treatment and chick was the experimental unit. Differences in BW gain, FI and T_b between heat exposed and control chicks during the period of heat exposure, and BW gain and FI during *Eimeria* infection were analysed with a PROC GLM for the effects of EST treatment, heat exposure, and their interaction. Pen was the experimental unit for BW gain and feed intake and chick was the experimental unit for T_b. Differences in the number of secreted oocysts per gram faeces during Eimeria infection were determined with a PROC GLM for the effects of EST treatment, heat exposure, day p.i., and their interactions, with chick as the experimental unit. A PROC MIXED for repeated measurements was used for overall BW and feed intake analysis during the experimental period to determine effects of EST treatment, heat exposure, age, room and their interactions, with pen as the experimental unit. The TOEPH structure was used to account for heterogeneous variance and was the best fit to this model. Data are presented as least square means ± s.e.m. in tables and figures, unless stated otherwise. If data were not normally distributed, a log transformation was performed before analysis. Non-significant interactions were excluded from analysis by stepwise deletion. Effects were considered significant at $P \leq 0.05$ and a trend at $0.05 < P < 0.10$.

RESULTS

Embryo development and chick quality

Incubation time in SI embryos was on average 8 h longer than in OI embryos ($P<0.001$; Table 1). Hatch of fertile was 78.9% in the SI treatment and 81.9% in the OI treatment and not affected by incubation treatment.

Chick length of 9 h old chicks was on average 0.2 cm shorter in SI chicks compared with OI chicks ($P=0.01$; Table 1). Navel condition was significantly worse in SI chicks than in OI chicks ($P<0.001$). Dissection of the 20 male chicks per treatment demonstrated a reduced relative heart weight in SI chicks compared with OI chicks (Table 1; $P<0.001$), but no differences were observed in YFBM and RY weight (Table 1). Plasma measurements demonstrated significantly lower T_3, but not T_4, levels in 9 h old SI chicks compared to OI chicks ($P=0.02$; Table 1).

Tonic Immobility and Manual Restraint Test

Tonic Immobility (TI) duration at 14 d of age did not differ between SI (88.9 ± 14.8 sec.) and OI (89.0 ± 13.0 sec.) chicks, neither the number of attempts needed to induce TI (1.34 ± 0.15 sec. in SI chicks vs. 1.41 ± 0.15 sec. in OI chicks).

The latency to vocalize and struggle during the manual restraint test, as well as the total number of vocalizations and struggles, was not affected by incubation treatment. Corticosterone levels did not differ between incubation treatments before the manual restraint, but immediately after the 5 min. restraint corticosterone levels in OI chicks (44.2 ± 2.1 ng/mL) were higher than in SI chicks (36.6 ± 2.2 ng/mL) ($P=0.017$)

Temperature Preference and Heat exposure

Temperature preference of chicks was not affected by incubation treatment at d 1 (OI= 29.90 ± 0.22°C and SI= 30.02 ± 0.22°C; $P=0.62$) and d 7 (OI= 27.37 ± 0.36°C and SI= 27.10 ± 0.36°C; $P=0.61$), but the preferred ambient temperature decreased with increasing age of the chicks. No differences were found between incubation treatments in BW gain during the 72 h period of heat exposure, nor in feed intake

Table 1. Effect of eggshell temperature (EST) treatment on incubation time and layer hatchling characteristics (LSmeans ± s.e.m.).

EST treatment[1]				
Item	Suboptimal	Optimal	s.e.m.	P-value
Incubation time (h)	497	489	0.5	***
Hatchling length (cm)	17.9	18.1	0.1	*
Hatchling BW (g)	43.0	42.9	0.4	n.s.
Navel score (%)				
1 (good)	13.7	34.6	-	***
2 (moderate)	57.1	48.9	-	n.s.
3 (bad)	29.2	16.5	-	*
Heart weight[2] (% BW)	0.49	0.58	0.01	***
YFBM[2,3] (g)	36.9	36.8	0.62	n.s.
RY[2,3] (g)	6.0	5.8	0.28	n.s.
T_3[2,3] (ng/mL)	0.86	1.11	0.05	*
T_4[2,3] (ng/mL)	4.19	4.52	0.20	n.s.

[1] Suboptimal= EST of 36.7°C from ED 0-7, 37.8°C from ED 8-14 and 38.9°C from ED 15-21; Optimal = EST of 37.8°C from ED 0-21.

[2] Measured in 20 male chicks per EST treatment, equally divided over hatching times.

[3] YFBM = yolk free body mass; RY = residual yolk; T_3 = triiodothyronine; T_4 = thyroxin.

*P<0.05; **P<0.01; ***P<0.001; n.s. = not significant

(OI-heat= 42.08 ± 0.77 g/chick/day and SI-heat= 40.65 ± 0.77 g/chick/day; P=0.20). Although heat exposure increased the Tb of chicks significantly (41.8°C) compared to non-exposed chicks (41.4°C) in the same period (P<0.001), no effect of incubation treatment on T_b during heat exposure was found. Moreover, incubation treatment also had no effect on plasma T_3, T_4, and corticosterone levels before heat exposure (t = 0 h) nor on levels after 72 h heat exposure (results not shown).

E. acervulina infection

Dissection of the intestines at d 3, 4, and 7 p.i. demonstrated intestinal lesions in the duodenum due to *E. acervulina*. There was a significant day effect (P<0.001), in which lesion severity increased from d 3 p.i. to d 4 p.i. and declined thereafter (Figure 1). SI chicks tended to have more severe duodenal lesions at d 3 p.i (P=0.09). This effect could mainly be attributed to increased lesion severity in SI chicks that were exposed to heat before the infection (incubation treatment*heat interaction; P=0.09). No significant differences in lesions between treatments were observed at d 4 p.i., and d 7 p.i.

Microscopically examination of faecal samples from the dissected chicks demonstrated that there was no *E. acervulina* oocyst production at d 3 p.i., and production started at d 4 p.i. (Figure 2). The amount of oocysts produced per gram faeces did not

differ at d 4 p.i. (P>0.05), but SI chicks produced significantly more oocysts per gram faeces than OI chicks at d 7 p.i. (P=0.02).

Daily BW gain and feed intake per chick after *E. acervulina* infection was divided into three periods, d 0-3 p.i, d 3-5 p.i., and d 5-7 p.i. (Table 2). The infection resulted in a reduced BW gain and feed intake from d 3-5 p.i. in all chicks, compared with the BW gain and feed intake from d 0-3 p.i. (P<0.001). However, chicks did not lose weight (Table 2). From d 5-7 p.i., BW gain and feed intake increased again.

From d 0-3 p.i., no interaction or differences were found between incubation and heat treatments in BW gain and feed intake. From d 3-5 p.i., there was no incubation*heat interaction but OI chicks tended to eat more (P=0.07) and gain more BW (P=0.08) than SI chicks. Moreover, heat exposed chicks gained more weight than control chicks (P<0.001), whereas FI between heat exposed and control chicks did not differ. From d 5-7 p.i., no effect of incubation treatment on BW gain and feed intake was found. Heat exposure before infection resulted in a higher FI but lower BW gain from d 5-7 p.i. (P<0.05).

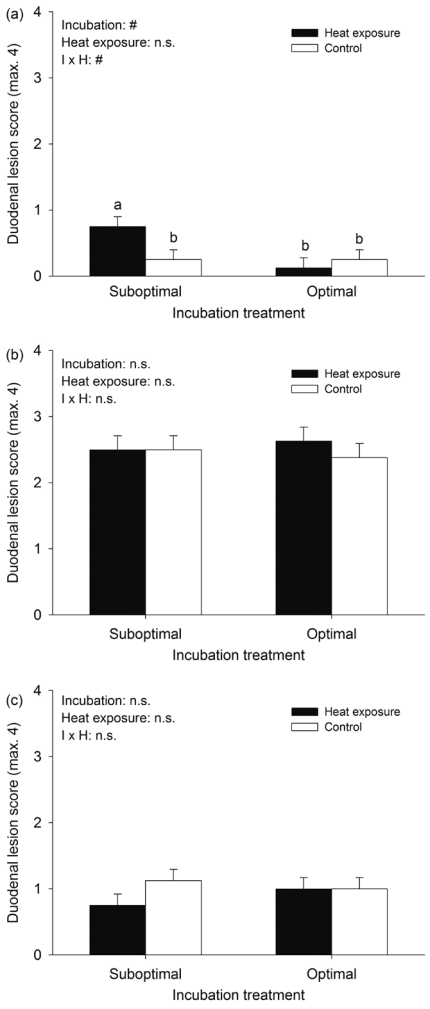

Figure 1. Duodenal lesion score at d 3 (a), d 4 (b) and d 7 (c) after *E. acervulina* infection in 36-day old layer chicks incubated at suboptimal or optimal eggshell temperatures (EST), that were either exposed to heat (35°C) for 72 h or kept under normal conditions (21.°C) preceding the infection (LSmeans ± s.e.m.).abLSmeans followed by different letters are significantly different (P<0.05). #P<0.10.

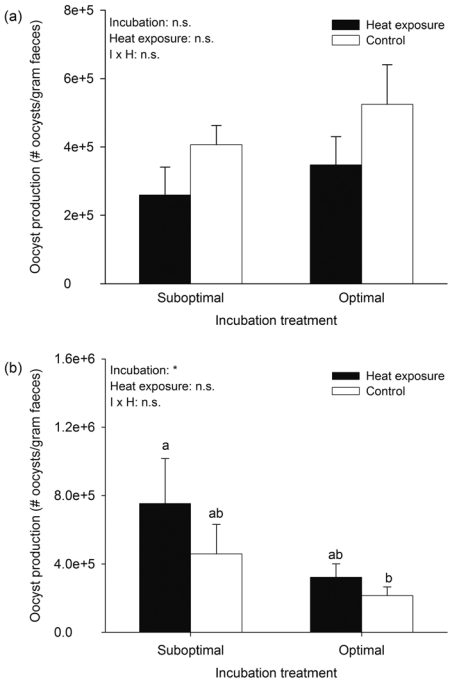

Figure 2. Oocyst production at d 4 (a) and d 7 (b) after *E. acervulina* infection (d 36 of age) in layer chicks incubated at suboptimal or optimal eggshell temperatures (EST), that were either exposed to heat (35°C) for 72 h or kept under normal conditions (21°C) preceding the infection (LSmeans ± s.e.m.). abcLSmeans followed by different letters are significantly different ($P<0.05$).*$P<0.05$.

Table 2. Average BW gain and feed intake per chick/day from d 0-3, d 3-5 and d 5-7 after *E. acervulina* infection in 36-day old layer chicks incubated at suboptimal or optimal eggshell temperatures, that were either exposed to heat (35°C) for 72 h or kept under control conditions (21°C) preceding the infection (LSmeans ± s.e.m.).

Days post E. acervulina infection							
Treatment		d 0-3		d 3-5		d 5-7	
Incubation[1]	Heat[2]	BW gain (g)	Feed intake (g)	BW gain (g)	Feed intake (g)	BW gain (g)	Feed intake (g)
Suboptimal	Heat exp.	18.2	40.6	9.7	28.5	16.2	50.2
	Control	21.3	41.5	5.2	29.7	18.8	46.4
Optimal	Heat exp.	21.3	42.1	10.6	31.8	14.1	49.9
	Control	20.1	42.1	5.2	30.1	17.7	47.0
	s.e.m.	3.7	0.8	0.9	1.0	1.1	1.3
P-value	Incubation (I)	n.s.	n.s.	#	#	n.s.	n.s.
	Heat (H)	n.s.	n.s.	***	n.s.	*	*
	I x H	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[1] Suboptimal= EST of 36.7°C from ED 0-7, 37.8°C from ED 8-14 and 38.9°C from ED 15-21; Optimal = EST of 37.8°C from ED 0-21.

[2] Heat exposure was from d 33-36 of age, starting and ending at 08.00 am.

0.05<P<0.10; *P<0.05; **P<0.01; ***P<0.001; n.s. = not significant

BW and Feed Intake during the experiment

No differences in body weight and feed intake between treatments were observed during the experiment (results not shown), apart from the above described differences during the *E. acervulina* infection. At the end of the experiment (d 61 of age) heat exposed OI chicks weighed 817.6 g ± 10.2 and heat exposed SI chicks weighed 807.2 g ± 10.2. In addition, control OI chicks weighed 813.71 g ± 10.2, whereas control SI chicks weighed 810.5 g ± 10.2.

DISCUSSION

The current study aimed to investigate whether suboptimal temperature during incubation of layer embryos affects their development and post hatch adaptive capacity to a model infection with *Eimeria acervulina*, under normal conditions and following a presumed stressful period of high ambient temperature.

Layer chickens exposed to suboptimal temperatures during incubation appeared to have a reduced ability to respond to *E. acervulina* at d 36 of age, regardless of whether they were exposed to heat before infection or kept under control temperatures. This was demonstrated by tendencies to lower feed intake and BW gain, more duodenal lesions and higher oocyst production after infection. At d 3 p.i., the percentage of chickens with lesions in the duodenum as a result of *Eimeria acervulina* (Lillehoj and Trout, 1996) was lower in the OI (18.8 %) than SI (50.0%) treatment. The higher percentage of chickens with lesions in the SI treatment was mainly caused by SI chickens that were exposed to heat before infection. This indicates that the duodenum of SI chickens, and in particular of SI*heat chickens, was more affected by *Eimeria acervulina* in the first phase of the infection. Intestinal lesions during *Eimeria* infections are caused by sporozoites, which are released after the *Eimeria* oocyst wall is cracked in the gizzard, and penetrate the intestinal epithelial cells where they undergo several reproductive phases in the tissue (Rose et al., 1996). These reproductive phases lead to most damage to the intestinal tissue. The severity of the intestinal lesions is a measure for the infection severity and is often related to a downward trend in weight gain (Reid and Johnson 1970). This is in correspondence with results in our study, where higher lesion scores on d 3 p.i. were followed by a tendency to lower feed intake and BW gain from 348 d 3-5 p.i. in SI chickens. Moreover, SI chickens had a significantly higher oocyst production at d 7 p.i. compared with OI chickens. Again, this effect was mainly due to higher oocyst production of SI*heat chickens. The output of oocysts in the faeces is a widely used method in disease diagnosis and also a measure for the infection severity (Chapman and Rayavarapu, 2007). Furthermore, it says something about the chickens' resistance to *Eimeria* (Dalloul et al., 2003), the higher the oocyst production, the lower the resistance to *Eimeria*. These observations all indicate that SI chickens had a lower capacity to adapt during the *Eimeria acervulina* infection than OI chickens. And in particular, based on the results of duodenal lesions and oocyst production, SI chickens exposed to heat before infection were most affected by *E. acervulina* in the first phase of infection.

We hypothesized that the reduced capacity of SI chickens, and in particular SI*heat chickens, to cope with *E. acervulina* might be due to several reasons. First, it may relate to developmental differences of the chickens. The effects of suboptimal incubation temperature on development in the present study were determined based on other studies (Hill, 2001; Molenaar et al., 2008), by measuring heart weight, chick

length, navel condition, BW, YFBM, and residual yolk weight. Our results demonstrated that suboptimal incubation temperature had a negative effect on embryonic growth and development. At hatch, relative and absolute heart weights were lower in SI chickens than in OI chickens. Furthermore, chick length and navel condition, which are indicators for chick quality (Wolanski et al., 2004; Fasenko and O'Dea 2008; Molenaar et al., 2008), were reduced in SI chickens. It is possible that these differences in chick quality were the result of metabolic differences of the embryos, since T3 levels at hatch were different between SI and OI chickens. Although there are several studies that demonstrated adverse effects of reduced chick quality on performance later on (Wolanski et al., 2004; Molenaar et al., 2008; Fasenko and O'Dea, 2008), in the current study we found no negative effects on BW development, feed intake and/or mortality in the post hatch period, but we also did not look at organ development at a later age. Therefore, it is possible that differences between chickens are only apparent in times of infectious challenges in which organs are affected, such as during the intestinal *E. acervulina* infection. Another challenging situation in which we observed differences between incubation treatments in the current study was the manual restraint. Immediately after the manual restraint 378 test, SI chickens had significant lower corticosterone levels than OI chickens, indicating a lower activity of the HPA axis (Braastad, 1998). This result is probably more related to the effects of prenatal development of physiological control systems (Dorner, 1974). Corticosterone levels can be interpreted as an adaptive response to stressful events (Martins et al. 2007). For example, free-living birds respond to environmental stresses by up-regulating corticosterone production (Martins et al. 2007). In our study, the lower corticosterone response of SI chickens after manual restraint might therefore be interpreted as a reduction in adaptive response to a stressor. This may consequently also affect their adaptive response to other stressors than the manual restraint, which would explain the reduced capacity to respond to *Eimeria*.
Second, differences between SI and OI chicks in coping with the *E. acervulina* infection might be more related to the availability of internal resources in order to respond to a challenge. In this context, the reduced ability of SI*heat chickens to cope with *Eimeria* might partly be due to differences in response to the heat exposure. Heat exposure, regardless of incubation treatment, resulted in a higher BW gain from d 3-5 p.i, but a lower BW gain (and higher FI) from d 5-7 p.i. Heat exposed chickens also had lower plasma T3 levels and higher T4 levels just before infection, compared to control chickens. Beside the role of thyroid hormones in metabolism, they are also known to be involved in the functioning of the immune system, especially in regulating lymphocyte reactivity (Klecha et al., 2006). Immune responses to *E. acervulina* are mostly cell dependent (Dalloul and Lillehoj, 2005) and therefore, immune modulation by changes in thyroid hormones might be a reason for the significant difference in BW gain in response to *Eimeria* between heat exposed and control chickens. Although the heat exposure did not result in differences in performance and endocrine response between SI and OI chickens, it is possible that SI chickens had more difficulties than OI chickens to maintain their Tb and metabolic rate at a normal level during the heat exposure. It is possible that when the heat stress was more extreme, with a higher temperature and/or longer duration, differences between SI and OI chicks in coping with the heat would be present. Especially because differences in thyroid hormone levels between SI and OI chicks were already found at hatch. Therefore the energy costs for maintenance during heat exposure could be higher

in SI chickens, which might have resulted in a trade off with immune function in the first phase of the *E. acervulina* infection (Moberg, 2000), also because inoculation of *E. acervulina* occurred immediately after heat exposure, which was probably still in the recovery phase for the chickens. Although this remains a speculation, it could explain why SI*heat chickens had more lesions and a higher oocyst production after infection than chickens of all other treatments.

In conclusion, exposure of layer embryos to a suboptimal incubation temperature in wk 1 and wk 3 of incubation reduced embryo development and post hatch corticosterone levels during manual restraint. Furthermore, suboptimal incubation tended to reduce the performance of chickens during *E. acervulina* and resulted in higher lesion scores and faecal oocyst numbers, especially when suboptimal incubation was combined with heat exposure preceding the infection. This demonstrates that suboptimal incubated chickens are less able to cope with an intestinal pathogen post hatch, especially after already stressful conditions of heat exposure. This is a disadvantage because environmental challenges can also occur consecutively in production systems. This study therefore emphasizes the importance of incubation temperature for layer embryos in the development of their ability to cope with post hatch (infectious) challenges.

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EFFECT OF EARLY FEEDING AND COLISTIN TREATMENT IN YOUNG LAYING HENS:

1. PERFORMANCE, INTESTINAL MICROBIOTA COMPOSITION AND RESPONSE TO EXTRA-INTESTINAL IMMUNE CHALLENGE

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ABSTRACT

Early feeding is known to influence immunity in poultry. Moreover, changes in microbiota composition, e.g. due to early feeding or use of antibiotics, may influence the ability to generate immune responses in times of infectious challenges, and thereby the adaptive capacity of poultry, however this was never directly investigated. Therefore, the present study aimed to investigate whether early feeding and early antibiotic treatment (Colistin) influence the intestinal microbiota composition and later life response of young layers to an immune challenge. Layers were used as model species and Colistin was used as a model substance to change (gram negative) bacterial colonization.

In a 2 x 2 factorial arrangement, newly hatched layers either received feed directly after hatch (F+) or were feed deprived for 72 h (F-), and were treated Colistin in their drinking water for 21 d (C+), or received normal drinking water (C-). Ileal and caecal samples were taken at 72 h, 21 d and 62 d of age to determine microbiota differences by Denaturing Gradient Gel Electrophoresis (DGGE). At d 62 and d 63 of age, all layers were intratracheally challenged with a 0.5 ml PBS solution containing 0.5 mg of lipopolysaccharide (LPS) + 0.1 mg of Humane Serum Albumin (HuSA) in the trachea.

Early fed layers had a higher overall feed intake and BW ($P<0.001$), a different microbiota composition in the ileum (72 h) and caecum(72 h, d 62), a lower BW gain after challenge and a higher LPS and KLH antibody titre than non-fed layers. Colistin treatment resulted in a changed microbiota composition in the caecum at 72 h and d 21 and a higher LPS titre compared to non-treated layers. We conclude that early feeding and/or Colistin treatment: 1) have longer lasting effects on intestinal microbiota composition (both), 2) lead to lasting differences in BW (early feeding), 3) increases specific LPS antibody response (both) and natural antibody response to KLH (early feeding) after a LPS/HuSA challenge, 4) have no effect on the adaptive humoral response determined by the HuSA titre.

Keywords: early feeding, Colistin, immune challenge, laying hens, microbiota composition

INTRODUCTION

At hatch, the chicken intestine is usually sterile and colonization of the gut with bacteria starts soon after the chick emerges from the eggshell (Gibbons, 1977). Previously it was demonstrated in mice that this colonization of the intestines with bacteria is important for immune activation in early life and also for the establishment of a proper functioning immune system in later life (Hrncir et al., 2008; Martin et al., 2010). Both the presence of bacteria in the intestines, as well as the composition and diversity of the intestinal microbiota in early life may play a role in immune development and functioning (Mulder et al., 2009; Inman et al., 2010). For this reason, early life modulation of the intestinal microbiota composition can influence immunity in later life and the risk for immune disorders (Bedford Russell and Murch, 2006; Croswell et al., 2009). A healthy intestinal microbiota and a properly functioning immune system are of special importance for production animals, such as laying hens, because they are housed under intensive rearing conditions, which make them vulnerable to the rapid spread of infections.

Sudden changes in the microbiota composition may occur due to internal factors such as hormonal shifts (Havenaar and Huis in't Veld, 1992), the mucosal immune system (Hansen et al., 2010) and external factors such as feed deprivation and antibiotic treatment (La Ragione et al., 2005). These factors may cause an unstable microbiota composition, which allows (opportunistic) pathogenic bacteria to colonize the gut (Berg, 1999). Although many studies mentioned refer to human and mouse models, the same occurs most likely in avian species.

Early feeding in layers, e.g. the provision of feed immediately after hatch, might play a significant role in intestinal microbiota colonization and immune development, which was previously suggested by Bar-Shira et al. (2005). Newly hatched layers in practice have no access to feed and water until arrival at the rearing farm, which may take up to 24-72 h. However, early feeding results in higher growth rates (Bar Shira et al., 2005), better intestinal development, and better development and maturation of the intestinal (Gut Associated Lymphoid Tissue; GALT) and systemic immune system (Noy et al., 2001; Juul-Madsen et al., 2004). Feed contains many live bacterial cells and conserved Microbiota Associated Molecular Patterns (MAMP's), such as lipopolysaccharide (LPS) (Hrncir et al., 2008). Because dendritic cells of the immune system continuously sample the gut, early exposure to these bacteria and MAMP's through early feeding will trigger the immune system and can help to influence immunity (Uni et al., 1998).

The microbiota composition of the chicken intestine in the first few weeks of life is dominated by gram negative *Enterobacteriaceae* and gram positive *Lactobacilli* (Barnes, 1980; La Ragione et al., 2005). It is therefore likely that early life changes in microbiota composition due to early feeding are related to these groups of bacteria. In a preliminary study we found that early life modulation of gram negative intestinal bacteria with the antibiotic Colistin (polymixin E) during early immune maturation (d 1-21 post hatch), influenced the cachectin response to intratracheally administered LPS and impaired the adaptive humoral immune responsiveness of young laying hens (Lammers et al., in preparation). Because inhibition of gram negative intestinal bacteria influenced immunity, we hypothesized that the effects of early feeding on performance and immunity could be related to the early colonization of gram negative bacteria in the intestines. Colistin is a bactericidal for most gram negative bacteria and is able to neutralize the biological activity of LPS. Moreover, Colistin

has the advantage that it is only locally effective in the intestine, because it is not taken up systemically when orally supplied (Zeng et al., 2010). Therefore, the antibiotic Colistin had the right properties, which enabled us to investigate a relation between early feeding, the gram negative bacterial population and immune responsiveness. Although the relation between early feeding, intestinal (gram negative) microbiota changes, performance and immunity in layers can easily be envisaged, it has, to our knowledge, never been investigated. Therefore, the objectives of the present study were to investigate: 1) whether early feeding and treatment with the antibiotic Colistin (as a model substance) indeed influence the intestinal microbiota composition, 2) the effects of early feeding and Colistin treatment on chicken development and adaptive response to an extra-intestinal non-infectious immune challenge (mimicked lung infection) and, 3) whether there was an interaction between early feeding and Colistin treatment on the above described effects. In an accompanying paper (Walstra et al., 2011b) the effects of early feeding and Colistin treatment on organ weights and response to an intestinal, infectious challenge as may occur in commercial poultry husbandry systems is reported.

MATERIAL AND METHODS

Ethics statement

All procedures in this study were carried out according to the recommendation of and following the approval of the Animal Care and Use committee of Wageningen University, the Netherlands. The Animal Care and Use committee of Wageningen University in the Netherlands therefore approved the present study and registered the study under approval ID 2009135.c.

Experimental design

The experiment was designed as a 2 x 2 factorial arrangement. Newly hatched layers either received feed directly after hatch or were feed deprived for 72 h, and were treated with the antibiotic Colistin (bactericidal for gram negative bacteria) in their drinking water for 21 d, or received drinking water without Colistin. At d 62 and d 63 of age, all layers were intratracheally (i.t.) challenged with PBS containing lipopolysaccharide (LPS) and Humane Serum Albumin (HuSA).

Incubation and Hatch

Eggs of a Lohmann Brown layer breeder flock (44 weeks; Verbeek Hatchery, Lunteren, the Netherlands) were randomly divided over two HT-combi incubators (HatchTech B.V., Veenendaal, The Netherlands). Incubation was performed at a constant eggshell temperature (EST) of 37.8°C, with 55-60% relative humidity (RH) and eggs were turned each hour. During embryonic day 19, 20 and 21, eggs were checked for hatching every three hours.

Housing, early feeding and Colistin administration

After hatch, layers were colour sexed and dry female layers were collected (n = 256), individually tagged (Swiftack ®), weighed and randomly assigned to one of four treatments. F+C+ and F+C- layers received feed and F-C+ and F-C- layers were feed deprived for 72 h. In addition, F+C+ and F-C+ layers received the antibiotic Colistin® (Polymyxin E; Dopharma B.V., Raamsdonksveer, the Netherlands) in their drinking water (4 mL/L water) until 21 days of age, whereas F+C- and F-C- layers

received drinking water without Colistin. Each treatment group (n = 56) was housed on wood shavings in a floor pen (200 x 200 cm).
At 72 hours post hatch, each layer was weighed again and 8 layers per treatment group (divided over hatching times) were collected for autopsy. Autopsy layers were decapitated and a 1 cm section of the ileum and caecum was collected and stored at -80°C for further analysis with DNA extraction, PCR and DGGE (section below). This procedure was repeated at d 21 and d 62 of age.
At d 6 of age, layers were transported to their rearing environment and divided over 32 floor pens (8 pens per treatment) in two different climate rooms. Six layers of the same treatment were housed per pen with a density of 4 birds/m2. Body weight and feed intake was determined weekly. Temperature in the climate rooms was 33°C at day 1 and gradually decreased until 20°C at day 35 until d 91. Light/darkness program was as follows: wk 1-2 16:8, wk 3 14:10, wk 4 12:12, wk 5 11:13, wk 6 10:14 and wk 7-13 9:15.

LPS/HuSA challenge

At d 62 and d 63 of age, all layers received an intra-tracheal (i.t.) challenge with 0.5 ml PBS containing 0.5 mg of LPS (L2880, Sigma Chemical Co., St. Louis, MO) + 0.1 mg of HuSA (A8763, Sigma Chemical Co.). The LPS and HuSA doses were given i.t. as a mixture, using a blunted needle. On d 1, 2, 3, 7, 10, and 14 post challenge, all layers and feed trays were weighed to determine BW gain and feed intake after the challenge.
Blood samples were taken from the wing vein of 4 layers per pen right before the LPS/HuSA challenge (d 0), and on d 3, 7, 14 and 21 after challenge for measurements of specific antibodies to LPS and HuSA and natural antibodies to KLH. Blood was centrifuged at 4750 rpm for 6 minutes and plasma was stored in -20°C for further processing.

Specific antibodies to LPS and HuSA

The specific antibody binding to LPS or HuSA was determined by and ELISA procedure previously described by Star et al. (2007). 96-well medium binding flat-bottomed ELISA-plates were coated with 4 μg/mL of *Escherichia coli* LPS (serotype O55:B5, Sigma Chemical Co., St. Louis, MO) or 4 μg/mL of HuSA (human albumin FRV ICN, Sigma Chemical Co.) and incubated at 4°C overnight. After washing the plates were filled with 100μl PBS containing 0.05 % Tween 20 and 0.5 % horse serum. Plasma samples were stepwise diluted (1:40, 1:160, 1:640, 1:2560) and subsequently incubated at room temperature for one hour. After washing, binding of the antibodies to LPS and HuSA was determined by using rabbit-anti-chicken IgG_{H+L} labelled peroxidase (1:20000 dilution, RACh/ IgG_{H+L}/PO; Nordic, Tilburg, the Netherlands) and plates were again incubated at room temperature for one hour. After washing, tetramethylbenzidine with 0.05 % H_2O_2 was added and 10 minutes later the reaction was stopped with 2.5 N H_2SO_4. Extinctions were measured at a wavelength of 450 nm with a Multiskan (Labsystems, Helsinki, Finland). Titres were calculated based on log_2 values of dilutions that had an extinction closest to 50 % of E_{max} (highest mean extinction of a standard positive sample on each plate).

Natural antibodies to KLH

The natural antibody binding to Keyhole Limpet Hemocyanin (KLH) was determined by an ELISA procedure as described in the previous section. 96-well medium binding flat-bottomed ELISA-plates were coated with 2 μg/mL of KLH (MP Biomedicals Inc., Aurora, Ohio, 44202). Plasma samples were diluted in four steps (1:40, 1:160, 1:640, 1:2560).

DNA extraction and purification

The method for DNA extraction was described by Nylund et al. (2010). DNA was extracted from 4 randomly selected ileum and caecum samples on day 3, 21 and 62 post hatch. Each sample was pre-treated with 500μl of 1× phosphate buffer solution(PBS), 500μl of 4% sodium dodecyl sulphate(SDS) and 10μl of 20 mg ml^{-1} Proteinase K , incubated at 55°C for an hour before the application of repeated bead beating (RBB) + Qiagen method (QIAamp® DNA stool mini kit, Qiagen GmbH, Hilden, Germany). Briefly, cell lysis was achieved by RBB in Precellys®24 (Bertin, Montigny-le-Bretonneux, FR) instrument, in the presence of 4% SDS, 500 mM NaCl, 50 mM Tris-HCL (pH 8), and 50 nM EDTA. Following RBB, samples were incubated at 95°C for 15 min, centrifuged and precipitated with 10 M ammonium acetate. Nucleic acids were subsequently recovered by precipitation with isopropanol. Nucleic acid pellets were washed with 70% ethanol, dried and re-suspended in 100 μl of TE buffer. Purification was attained by adding 2 μl (10 mg/ml) RNase and incubate the samples for 15 min at 37°C. Next, 15μl proteinase K and 200 μl Buffer AL was added, followed by the use of QIAamp columns to complete purification.

PCR and Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

DNA primers GC-968-f (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA CCT TAC-3') and 1401-r (5'- CGG TGT GTA CAA GAC CC-3') were used to amplify the V6 to V8 regions of the bacterial 16S ribosomal RNA (rRNA) gene (Nübel et al., 1996). A GoTaq® polymerase kit from Promega (Madison, WI, USA) was used to perform the PCR. Each PCR mixture (50 μl) contained 1.25 U of GoTaq® DNA polymerase, Green GoTaq® reaction buffer containing 1.5 mM MgCl2, 0.2 mM of each deoxynucleoside triphosphate, 0.2 μM of the primers, 1 μl of DNA solution (~1 ng/μl) and UV-sterilized water. Amplification of the samples occurred in a thermocycler T1 (Whatman Biometra, Gottingen, Germany) using the following program: pre-denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 40 sec, extension at 72°C for 60 sec and final extension at 72°C for 5 min. The size of the PCR products was determined by electrophoresis on a 1 %(W/v) agarose gel containing ethidium bromide.

The PCR amplicons were separated by denaturing gradient gel electrophoresis (DGGE, Muyzer et al.,1993), using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, samples were loaded onto 8% polyacrylamide gels with a denaturant gradient of 30–60% (100% was defined as 40% formamide and 7 M urea), pre-run for 5 min at 200 V, and subsequently electrophoresed at 85 V for 16 h at 60°C. Gels were developed by silver staining using the method of Sanguinetti et al. (1994), scanned at 400 d.p.i., and further analysed by BioNumerics 4.5 software (Applied Maths).

Statistical Analysis

For statistical analysis we used SAS software (SAS Institute Inc., Cary, NC, USA). Differences in body weight and feed intake over the experimental period were analysed with a PROC MIXED for repeated measurements for the effects of feed, Colistin, day and their interaction with climate room as random effect. Body weight was averaged per pen in SAS before analysis. BW gain after LPS/HuSA challenge was analysed from d 0-1, d 1-2 and d 2-3 post challenge with a PROC GLM for the effects of feed, Colistin and their interaction with climate room as random effect. LPS, HuSA and KLH antibody titres were analysed with a PROC MIXED for repeated measurements for the effects of feed, Colistin, day and their interaction with climate room as random effect. LPS titre data was not normally distributed and therefore a log(10) transformation was performed before analysis. Data are presented as least square means ± s.e.m. in Tables and Figures, unless stated otherwise. Non-significant interactions were excluded from analysis by stepwise deletion, except for the interaction between feed and Colistin treatment. Effects were considered significant at $P \leq 0.05$ and a trend at $0.05 < P < 0.10$. Pearson product moment correlation based on densitometric curves was used to assess the similarity of DGGE patterns obtained for different samples (Zoetendal et al., 2001; Fromin et al., 2002). Multivariate analysis was applied for DGGE data interpretation. In order to relate changes in bacterial composition to the environmental variables, redundancy analysis (RDA), as implemented in CANOCO 4.5 software package (Biometrics, Wageningen, the Netherlands), was used. RDA is a multivariate direct gradient analysis method that allows ordering of samples and taxa (i.e. DGGE bands) considering that species have linear relationships to environmental variables (Lepš and Šmilauer, 2003). Treatments (feed and Colistin) and sampling day (72 h, d 21 and d 62) were introduced as nominal environmental variables. Monte Carlo permutation with forward selection was applied to test for significance of the effect of the different treatments and sampling time on microbiota profiles. The effect of the environmental variables was considered significant at $P < 0.05$. Diagrams were plotted as biplots using CanoDraw for Windows.

RESULTS

Microbiota composition in the caecum

At 72 h, no interaction was found between feed and Colistin on the microbiota composition in the caecum. Early fed layers differed in microbiota composition from non-fed layers ($P=0.002$) and Colistin treated layers differed in microbiota composition from non- treated layers ($P=0.034$; Figure 1a). At d 21, no effects ($P>0.10$) of were found of early feeding and Colistin treatment, nor their interaction, on the microbiota composition of the caecum (Figure 1b). At d 62, a significant interaction between feed and Colistin on microbiota composition was observed. ($P=0.004$) (Figure 1c). Early fed layers differed in microbiota composition from non-fed layers ($P=0.002$) and Colistin treated layers differed in microbiota composition from non-treated layers ($P=0.004$).

Microbiota composition in the ileum

At 72 h, no interaction between feed and Colistin on microbiota composition in the ileum was observed. Early fed layers differed in microbiota composition from non-fed layers ($P=0.002$), but no effect of Colistin treatment on microbiota composition at 72 h was found (Figure 2a). At d 21 and d 62, no interaction between

feed or Colistin, nor an effect of feed and Colistin separately on the microbiota composition was observed (Figure 2b and 2c).

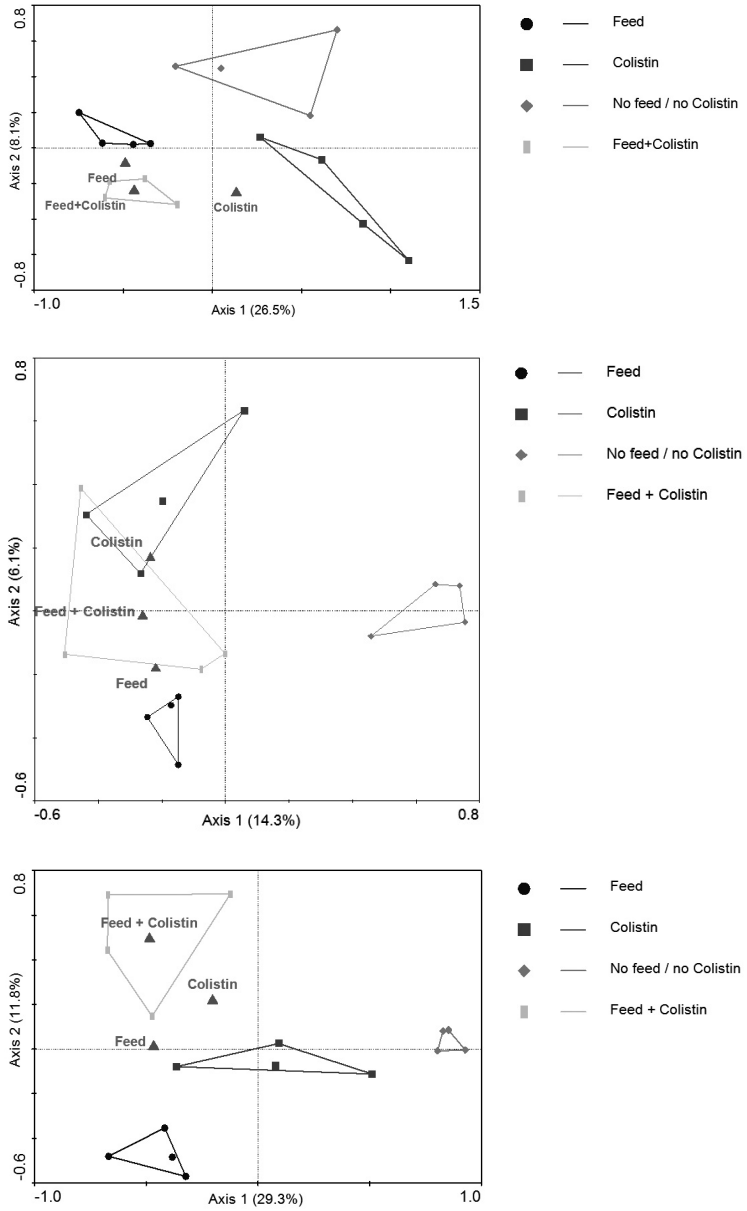

Figure 1. Ordination biplot for RDA analysis of DGGE profiles of caecal bacteria at 72 hours (A), day 21 (B) and day 62 of age (C). Nominal environmental variables feed, Colistin, and their interaction, are represented by triangles (▲). Samples are grouped by treatment: F+C- (•); F-C+ (■); F+C+ (▮); F-C-, (◆). Each symbol represents one animal. Both axis explain 34.6% of the total variance in the dataset for (A), 20.4% for (B), and 41.1% for (C).

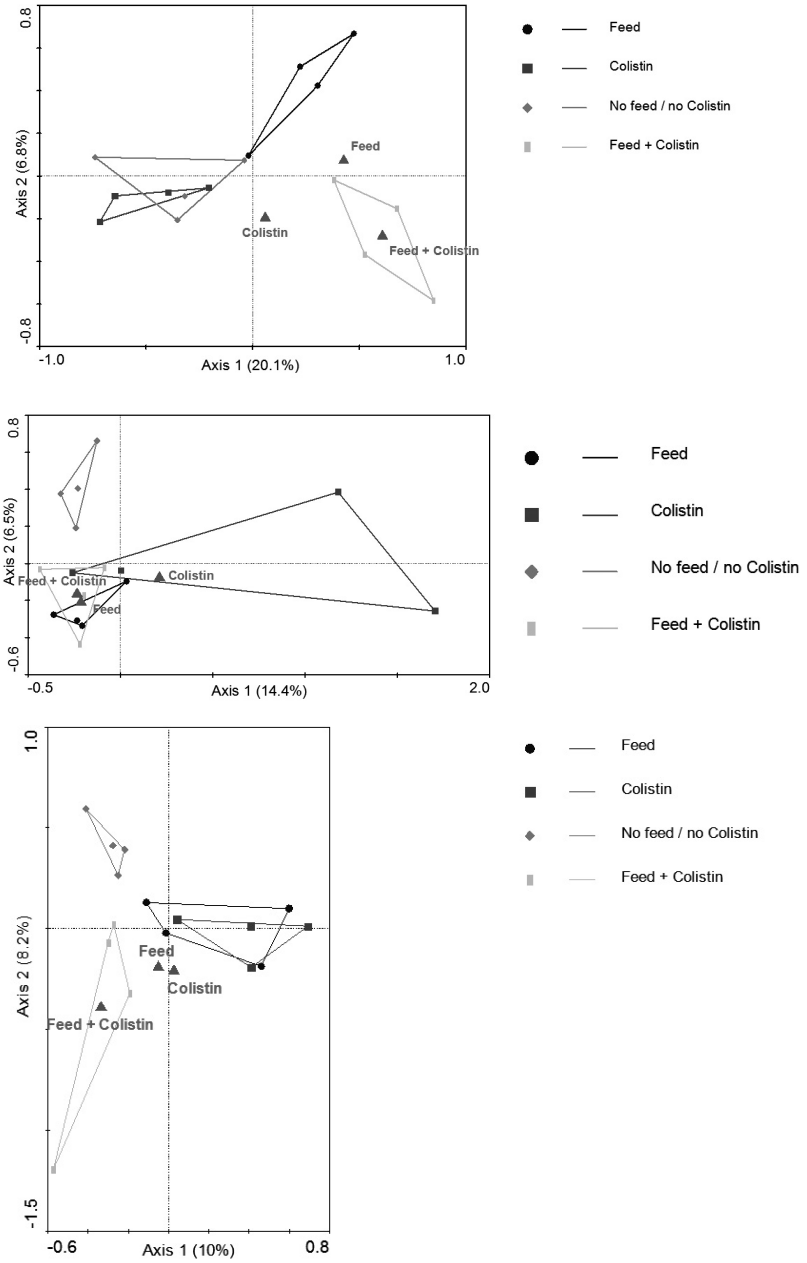

Figure 2. Ordination biplot for RDA analysis of DGGE profiles of caecal bacteria at 72 hours (A), day 21 (B) and day 62 (C). Nominal environmental variables feed, Colistin, and their interaction, are represented by triangles (▲). Samples are grouped by treatment: F+C- (•); F-C+ (■); F+C+ (▮); F-C-, (◆). Each symbol represents one animal. Both axis explain 26.9% of the total variance in the dataset for (A), 20.9% for (B), and 18.2% for (C).

Body weight and feed intake overall

No interaction was found between early feeding and Colistin treatment for body weight and feed intake. Early fed layers had a higher average BW (600.5 ± 5.69 g) and feed intake (50.1 ± 0.49) than non-fed layers (577.8 ± 5.69 g for BW and 49.2 ± 0.49 for feed intake) over the complete experimental period (P<0.001) and also differed significantly from non-fed layers when analysed per day from 72 h until 76 days post hatch for BW and from 72 h until d 43 for feed intake (results not shown). Colistin treatment had no effect on BW and feed intake during the experimental period.

BW gain LPS/HuSA challenge

BW gain after the LPS/HuSA challenge was divided in three periods, from d 0-1, d 1-2 and d 2-3 post challenge. From d 0-1 post challenge (Figure 3), a tendency towards a feed x Colistin interaction (P=0.07) was found in which F+C- layers lost 12.7 g (± 4.1) and differed from all other treatments. F+C+, F-C+ and F-C- layers all gained weight in the same period (1.9 g, 1.5 g and 2.5 g respectively) and did not differ. No interaction between feed and Colistin treatment was found for BW gain in the period from d 1-2 post challenge. Moreover, BW gain from d 1-2 post challenge was not affected by feed or Colistin treatment (Figure 3). No interaction was found between Feed and Colistin treatment for BW gain from d 2-3. In this period, BW gain tended to be affected by feed treatment (P=0.09). Early fed layers lost weight (-1.3 ± 7.9 g), whereas non-fed layers gained 3.6 ± 7.9 g weight. No effects of Colistin were found in this period.

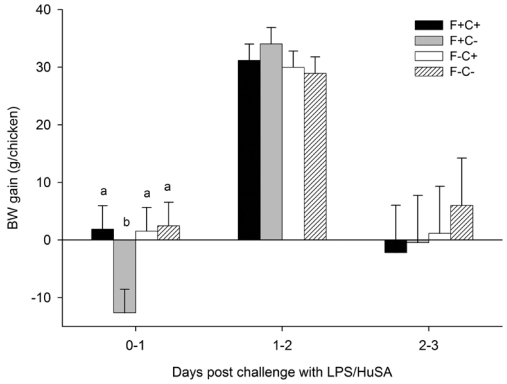

Figure 3. Body weight gain per chicken from d 0-1, d 1-2, d 2-3 after two intratracheal challenges with 0.5 ml PBS containing 0.5 mg of LPS and 0.1 mg of HuSA inoculation at respectively 62 d of age (d 0 post challenge) and 63 days of age (d 1 post challenge). Treatments represent layers that were fed immediately after hatch (F+) or 72 h later (F-), and were treated with the antibiotic Colistin for 21 d (C+) or were not treated (C-). Data represent least square means and s.e.m. Bars marked with different letters represent significant differences between treatment groups (P < 0.05).

Specific antibody response to LPS and HuSA

Total antibody responses to LPS were determined at d 0, 3, 7, 14 and 21 p.i. and are presented in Figure 4. Overall, a feed x Colistin interaction was found (P=0.02) in which

F+C+ layers had a higher average LPS titre than all other treatments, also when analysed per day. Average LPS titre of F+C-, F-C+ and F+C layers did not differ from each other (*P*>0.05). Total antibody responses to HuSA were measured at d 0, 3, 7, 14 and 21 p.i. Overall and analysed per day we observed no interaction between feed and Colistin, and also no effects of feed and Colistin treatment on antibody responses to HuSA (Figure 5).

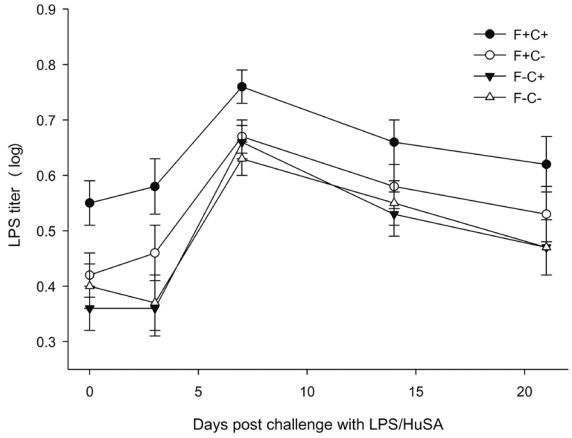

Figure 4. LPS antibody titre after two intratracheal challenges with 0.5 ml PBS containing 0.5 mg of LPS and 0.1 mg of HuSA inoculation at respectively 62 d of age (d 0 post challenge) and 63 days of age (d 1 post challenge). Treatments represent layers that were fed immediately after hatch (F+) or 72 h later (F-), and were treated with the antibiotic Colistin for 21 d (C+) or were not treated (C-). Data represent least square means and s.e.m.

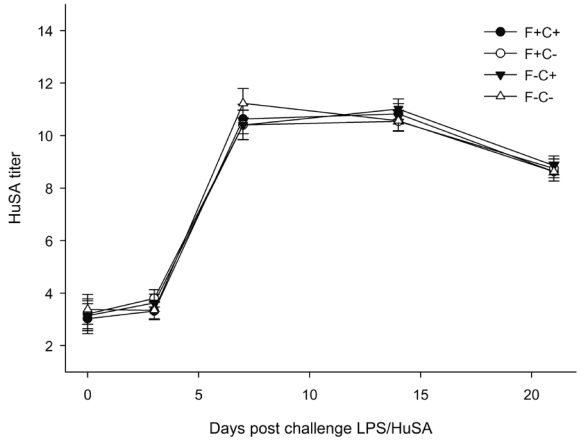

Figure 5. HuSA antibody titre after two intratracheal challenges with 0.5 ml PBS containing 0.5 mg of LPS and 0.1 mg of HuSA inoculation at respectively 62 d of age (d 0 post challenge) and 63 days of age (d 1 post challenge). Treatments represent layers that were fed immediately after hatch (F+) or 72 h later (F-), and were treated with the antibiotic Colistin for 21 d (C+) or were not treated (C-). Data represent least square means and s.e.m.

Natural antibody response to KLH

On average, feed and Colistin tended to interact (*P*=0.08) on KLH titre, in which F+C+ layers had a higher KLH titre than F+C- layers, whereas F-C+ layers had a lower KLH titre than F-C- layers. Early fed layers had a higher average natural antibodies to KLH than non-fed layers, but only differed significantly at d 7 when analysed per day (*P*=0.02). Colistin treatment had no effect on natural antibody titre to KLH (Figure 6).

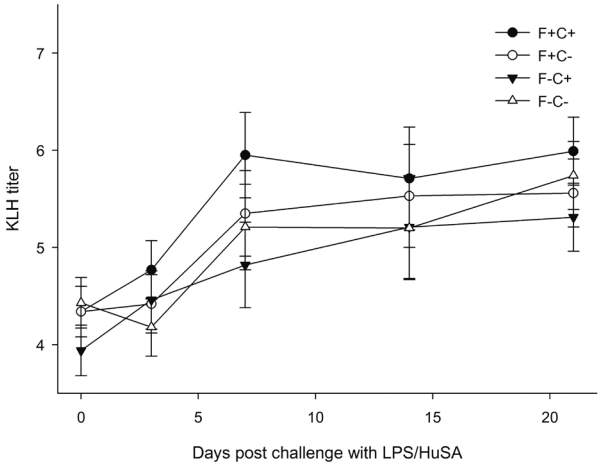

Figure 6. Natural antibody titre to KLH after two intratracheal challenges with 0.5 ml PBS containing 0.5 mg of LPS and 0.1 mg of HuSA inoculation at respectively 62 d of age (d 0 post challenge) and 63 days of age (d 1 post challenge). Treatments represent layers that were fed immediately after hatch (F+) or 72 h later (F-), and were treated with the antibiotic Colistin for 21 d (C+) or were not treated (C-). Data represent least square means and s.e.m.

DISCUSSION

The early post hatch period is critical for the establishment of a stable and healthy intestinal microbiota (Yegani and Korver, 2008). The process of intestinal colonization already starts immediately after hatching and continuous to a relatively stable status as the animal ages (Verstegen et al., 2005). Apajalahti et al. (2002) demonstrated that one day post hatch the ileum and caecum of broiler layers already have bacterial densities of 10^8 and 10^{10} cells per gram of digesta, respectively.

In the present study, the effect of early feeding (0-72 h) and Colistin treatment (d 1-21) on the microbiota composition were investigated in the chicken ileum and cecum, and subsequently the adaptive response to an immune challenge model with LPS/HuSA was determined. The ileum and caecum are colonized by bacteria early in life and both are important for the (development of the) immune system of chickens (La Ragione et al., 2005). Changes in early microbiota colonization and composition, for example due to early feeding or Colistin treatment, may therefore influence the response of layers when exposed to infectious challenges later in the rearing period .

Three time points were chosen in the present study to investigate differences in intestinal microbiota composition of the ileum and caecum: 1) 72 h post hatch,

which was the end of the early feeding contrast, 2) 21 days post hatch, which was the end of the Colistin treatment and, 3) 62 days post hatch, immediately before the LPS/HuSA challenge. The results of DGGE analysis of intestinal samples demonstrated that early feeding from 0-72 h post hatch influenced the microbiota composition of both the caecum and ileum. Moreover, Colistin treatment resulted in changes of the microbiota composition in cecum, but not in ileum. This confirms our hypothesis that both feed and Colistin treatment influence the early life colonization and composition of the intestinal microbiota. Because feed is not sterile and contains many bacteria (Uni et al., 1998), early fed layers will be exposed to different bacteria than non-fed layers which only ingested or inhaled bacteria from the home pen environment and not from the feed. Moreover, feed provides nutrients to the bacteria, which will stimulate their growth. Not all bacterial species may benefit equally well to the available nutrient molecules present in the feed. On the other hand, Colistin is an antibiotic that is bactericidal for gram negative bacteria, and suppression of these bacterial species probably results in more opportunities for other bacterial species, such as gram-positive bacteria, to colonize. Colistin treated layers are therefore also likely to differ in microbiota composition from non-treated layers.

The function of the ileum is mainly nutrient absorption, while the caecum is involved in bacterial fermentation. Since these regions function differently and provide different environments, different types of bacteria will colonize them and distinct microbiota will develop (Gong et al., 2002). It can therefore be expected that manipulation of the microbiota with early feeding or Colistin treatment has a different outcome in both intestinal regions. For the ileum we found a difference between early fed and non-fed layers in microbiota composition at 72 h of age, however not on the longer term. The ileum is considered important for immune modulation, as Peyer's Patches, which are also part of the GALT, are mainly located in the chicken ileum. Therefore, early life changes in the microbiota composition of the ileum (i.e. due to early feeding) may result in alterations in the immune response and subsequently the response to infectious challenges. In the cecum, early feeding and Colistin treatment affected the microbiota composition at 72 h post hatch and on 62 d post hatch, whereas no effect of early feeding and Colistin treatment on microbiota composition was found at d 21 post hatch. However, although not significant, the clustering analysis of DGGE profiles using UPGMA with BioNumerics for the data at d 21 demonstrated that all samples of layers receiving early feed grouped within a distinct cluster comparing to the layers without early feed (data not shown). Thus, dependent on the area of the intestines, both early feeding and Colistin influence the microbiota composition, and may affect the intestinal microbiota composition until d 62 of age.

These results demonstrate that a relative short period of feed restriction (72 h), and a disruption of the microbiota colonization with Colistin for 21 days in early life have longer lasting effects (until d 62 of age in the present study) on the microbiota composition of the caecum. This may subsequently affect the capacity of layers to respond to an immunological challenge, as the caecum is an important part of the intestines for the immune response. Caecal tonsils constitute the largest collection of GALT in the chicken (Yun et al., 2000), which contain both B- and T-cells and are thus involved in antibody production and cell mediated immune responses (Yun et al., 2000). Organized lymphoid tissue in caecal tonsils is not present at the time of hatching but develops shortly thereafter and immunological maturation and overall size

are dependent on the degree of antigenic stimulation in the intestine (Del Cacho et al., 1993). Because feed is not sterile and contains many (harmless) antigens, the provision of feed immediately post hatch may not only affect the microbiota composition of the caecum but may also contribute to the maturation of the caecal tonsils. Moreover, by providing nutrients for the bacteria already present in the intestines, early feeding may also influence the rate of subsequent colonization and the density of bacteria. This may ultimately result in a larger (bacterial) trigger to start immune maturation as well.

The early intake of feed is accompanied by rapid development of the gastrointestinal tract and associated organs (Bar-Shira et al., 2005; Yegani and Korver, 2008). The lack of access to feed until 72 h post hatch leads to a depression in performance of the chicken which may not be overcome at a later stage in life (Uni et al., 1998; Halevy et al., 2000). Although most studies on early feeding were performed in broilers, the current study demonstrated that the lack of feed access until 72 h post hatch also leads to a depression in feed intake until d 43 of age and subsequently in body weight development of laying hens, until 76 days of age. There may also be a role for the microbiota composition in the difference in performance between early fed and non-fed layers, as it was also previously demonstrated that diet-induced changes in gut microbiota may result in subsequent improvement in poultry performance (Torok et al., 2008).

Our results demonstrated that after the challenge with a respiratory infection model of LPS / HuSA, early fed layers had a lower BW gain. Early fed layers lost weight from 0-24 h, but this was mainly caused by F+C- layers. From previous studies it is known that exposure to LPS induces the cachectin activities of acute phase proteins such as interleukin (IL)-1 and IL-6 or tumor necrosis factor (TNF)a- like substances. Cachectins induce a state of anorexia, in which the animal loses BW (Parmentier et al., 1998). From this point of view, it seems that early fed, and especially F+C- layers are more responsive to LPS exposure based on BW gain immediately after challenge, seem to have a higher cachectin activity than layers from the non-fed groups.

We hypothesized that Colistin treated layers would have a higher BW response to LPS, as Colistin is an antibiotic with a gram negative spectrum, and therefore Colistin treated layers were most likely exposed to reduced numbers of gram negative bacteria before d 21. However no effects of Colistin treatment on BW gain after challenge were found. There is a significant period of time between the end of Colistin treatment (d 21) and the LPS/HuSA challenge (d 62) for C+ animals to be exposed to LPS. Moreover, LPS levels in the stable are higher as the layers grow older, for example due to higher faeces production and dust levels. These time effects may be a reason for our observation that Colistin treatment does, in contrast to our preliminary study, not affect LPS induced body weight loss after the LPS/HuSA challenge.

If we look at specific T-cell independent antibody titre to LPS, both early fed and Colistin treated layers had an overall higher LPS antibody titre than non-fed and non-treated layers, respectively, which indicates a higher immune responsiveness to LPS in early fed and Colistin treated layers. For both effects, the higher titre could mainly be attributed to F+C+ animals. Moreover, when we look at the natural antibody response to KLH, which is an exo-antigen that the layers most probably have not encountered before during life and therefore says something about their innate immunity, early fed layers had higher average natural antibody binding to

KLH. Previously it was demonstrated that a high natural antibody titre to KLH, i.e. high innate immunity, was related with a higher probability to survive the first laying period (Star et al., 2008), and with disease resistance (Parmentier et al., 2004). Our results may therefore indicate that early feeding has a positive effect on survival and disease resistance, however, this relation still needs to be directly investigated.

Although we found differences between our early feeding and Colistin treatment on antibody responses to KLH and LPS, the antibody titre to HuSA was not influenced by any of the four treatments. This suggests that despite the observed differences in microbiota composition, the T-cell dependent humoral immune response seems to be less affected than the T-cell independent and innate antibody levels. This is in contrast with previous studies. Bar-Shira et al. (2005) investigated the effects of early feeding on immune responsiveness in very young broilers (< 14 days of age) and they found an impaired adaptive immune response. They suggested that the microbiota composition may play a role in these effects. In our preliminary study (Lammers et al., in preparation) Colistin was also used to change microbiota composition in young layers (< 9 weeks) and in that study, Colistin treatment affected the adaptive humoral immune responses. In our study, layers were older and immunologically matured. We therefore suggest that the microbiota composition may particularly influence the adaptive immune responses in the period that the immune system of the chicken is still developing. However, this remains a hypothesis that requires further investigation.

The present study is, to our knowledge, the first study that investigates the effect of early feeding on changes in microbiota composition at different points in time, and subsequently the adaptive capacity of layer layers to a model non-infectious challenge by determining antibody responses. We can conclude that early feeding and/or Colistin treatment: 1) have longer lasting effects on intestinal microbiota composition (both), 2) lead to lasting differences in BW (early feeding), 3) increases specific LPS antibody response (both) and natural antibody response to KLH (early feeding) after a LPS/HuSA challenge, 4) have no effect on the adaptive humoral response determined by the HuSA titre.

This finding is especially important with regard to early feeding, given the fact that newly hatched layers in commercial circumstances do not have access to feed in the first 24-72 h post hatch. The results in this study demonstrated that a fairly short period (0-72 h) of feed provision post hatch can prime layer layers in such a way that their growth and immune response later in life is altered, in which the observed differences in intestinal microbiota composition are likely to play an important role. Therefore, providing feed immediately post hatch may be an effective tool to change the later life adaptive responses of (layer) layers to infectious challenges.

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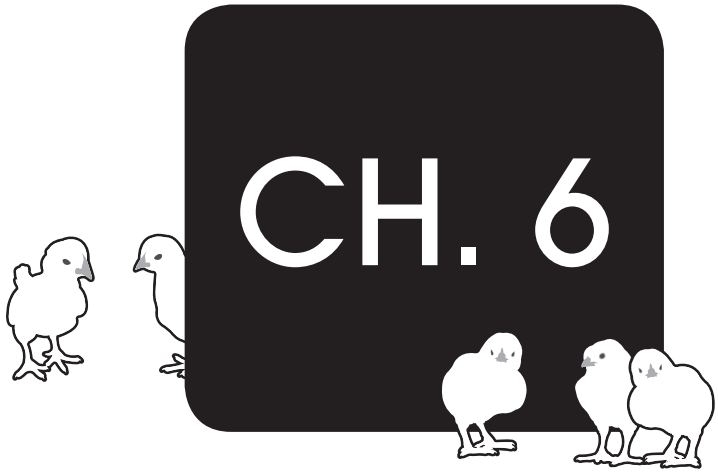

EFFECT OF EARLY FEEDING AND COLISTIN TREATMENT IN YOUNG LAYING HENS:

2. ORGAN WEIGHTS AND ADAPTIVE RESPONSE TO *EIMERIA ACERVULINA*

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Manuscript in preparation

ABSTRACT

Feed availability immediately post hatch is known to stimulate chicken development and development and maturation of the immune system. Feed contains many (harmless) bacteria and can thereby influence microbial composition in the intestines. As a stable, healthy intestinal microbiota is important for adequate immune development of chickens, changes in microbial composition, e.g. due to early feeding or antibiotic treatment, might influence the adaptive response of chickens during infectious challenges they may encounter in commercial husbandry systems.

The present study aimed to investigate whether early feeding, and changes in microbiota composition by treatment with the gram negative spectrum antibiotic Colistin, influence organ development post hatch and the adaptive response of young layers to an infectious challenge with *Eimeria acervulina*. Layers were used as model animals and Colistin as a model substance to change (gram negative) bacterial colonization. In a 2 x 2 factorial arrangement, newly hatched layers either received feed directly after hatch (F+) or were feed deprived for 72 h (F-), and were treated with Colistin in their drinking water for 21 d (C+), or received normal drinking water (C-). At d 27 of age, all chickens were inoculated with a 1 mL PBS solution containing 25.000 *E. acervulina* oocysts. At 72 h post hatch, organ weights were determined and BW gain, feed intake, oocyst production per gram faeces (OPG), intestinal lesions and antibody titre were determined after *E. acervulina* inoculation. Early feeding, but not Colistin treatment, resulted in higher BW, heart, liver and bursa weight at 72 h. Moreover, early feeding resulted in a lower BW gain, higher OPG and antibody titre, but lower lesion scores in the intestines after *E. acervulina*, and early fed chickens remained heavier throughout infection. Colistin treatment reduced antibody titre and the BW gain of non-fed chickens. From these results we can conclude that early feeding is positive for development, but also that early feeding and Colistin treatment result in changed adaptive responses to an *Eimeria acervulina* infection, indicating that the microbiota composition in early life influences the adaptive capacity of layers.

Keywords: adaptive response; Colistin; early feeding; *Eimeria* challenge; laying hens; microbiota composition

INTRODUCTION

Infectious diseases are a common threat for laying hens in the rearing period and it is therefore important that young laying hens already have a well-developed adaptive capacity. The adaptive capacity of an animal can be defined as the capacity to respond to an environmental stressor (e.g. pathogen) with the appropriate behavioural, physiological and immunological adjustments in order to maintain performance, health and welfare. It was previously demonstrated that early life conditions influence the adaptive capacity later in life (Walstra et al., 2010) and can thereby contribute to the ability to cope with infectious diseases. One example of an early life condition is the provision of feed directly after hatch. For laying hens, which have a longer rearing and production period than broilers and are likely to be exposed to a variety of infectious threats in these periods, providing early feeding may be a useful tool to improve their adaptive capacity to cope with these threats. However, this has to our knowledge, never been investigated.

Laying hens in commercial husbandry systems have no access to feed and water from hatch until arrival at the rearing farm, which might take up to 24-72 h due to variation in hatching time and logistic reasons (Dibner et al., 1998; Bar-Shira et al., 2005). However, previous studies in broilers demonstrated that when feed is immediately provided after hatch (i.e. early feeding), higher growth rates, better intestinal development, and a better development and maturation of the intestinal (Gut Associated Lymphoid Tissue; GALT) and systemic immune system are observed (Dibner et al., 1998; Noy et al., 2001; Juul-Madsen et al., 2004; Bar-Shira et al., 2005). It can easily be envisaged that the capacity to respond to infectious challenges will also be positively influenced by early feeding (Dibner et al., 1998).

The positive effects of early feeding may be related to early microbial colonization of the chicken intestines (Walstra et al., accompanying paper 2011a). At hatch, the chicken intestine is usually sterile but colonization of the gut starts as soon as the chicken hatches from the eggshell. Bar-Shira et al. (2003) demonstrated that immune activation also starts immediately after hatch, as soon as the chickens is exposed to environmental antigens and feed. Together with the fact that feed contains many live bacterial cells of different species, it functions also as a nutrient source for the developing microbiota. Therefore, it can easily be envisaged that early feeding can influence the bacterial colonization process of the intestines, which was also suggested by Bar-Shira et al.(2005). One of the first colonizing bacterial species are the facultative anaerobic gram negative *Enterobacteriaceae* and gram positive *Lactobacilli* (Barnes, 1980; La Ragione et al., 2005). It is therefore possible that early feeding will influence the growth of the these first colonizing bacteria.

Changes in microbiota composition can directly influence the immune system, and thereby the ability of chickens to respond to infections, as dendritic cells of the immune system are continuously sampling the gut and early exposure of dendritic cells to different intestinal bacteria may stimulate the development and maturation of the immune system and helps to create a wider antibody repertoire (Uni et al. 1998). Therefore, an optimal composition of the intestinal microbiota may positively influence the immune system, which will contribute to the adaptive capacity during infectious threats. We therefore hypothesized in the present study that changes in colonization of intestinal gram negative bacteria are important for immunity later in life and that the effects of early feeding on development may be linked to changes in the number and/or composition of the gram negative microbiota.

Therefore, the objectives of the present study were to investigate: 1) whether early feeding and treatment with the antibiotic Colistin (as a model substance to change gram negative bacterial colonization), influence development of young laying hens, 2) whether early feeding and Colistin treatment affect the later life capacity of young laying hens to cope with an infectious challenge as may occur in commercial husbandry systems, and 3) whether there was an interaction between early feeding and intestinal gram negative bacteria on the above described effects. Because both early feeding and Colistin treatment act on the mucosal surface of the gut, we used a mucosal infection model to challenge the adaptive capacity: the intestinal parasite *Eimeria acervulina.* In an accompanying paper (Walstra et al., 2011a), effects of early feeding and Colistin treatment on microbiota composition changes in ileum and caecum, performance, and response to a non-infectious extra intestinal immune challenge of young laying hens are reported.

MATERIAL AND METHODS

Ethics statement

The Animal Care and Use committee of Wageningen University in the Netherlands approved the present study and registered the study under approval ID 2009135.c.

Experimental design

The experiment was designed as a 2 x 2 factorial arrangement. Newly hatched chickens were either given feed *ad libitum* directly after hatch or feed was withheld from chickens for 72 h, and chickens were treated with Colistin in their drinking water from d 0-21 post hatch, or received drinking water without Colistin. At d 27 of age, all chickens were inoculated with *Eimeria acervulina* in the crop.

Incubation and Hatch

Eggs of a Lohmann Brown layer breeder flock (44 weeks; Verbeek Hatchery, Lunteren, the Netherlands) were randomly divided over two HT-combi incubators (HatchTech B.V., Veenendaal, the Netherlands). Incubation was performed at a constant eggshell temperature (EST) of 37.8°C, with 55-60% relative humidity (RH) and eggs were turned each hour. During embryonic day 19, 20 and 21, eggs were checked for hatching every three hours.

Housing, early feeding and Colistin administration

After hatch, chickens were colour sexed and dry female chickens (n = 256) were collected, individually tagged (Swiftack ®), weighed and randomly assigned to one of four treatments (Table 1). Each treatment group (n = 64) was housed on wood shavings in a floor pen (200 x 200 cm), where F+C+ and F+C- chickens received feed immediately and F-C+ and F-C- chickens were non-fed for 72 h and received feed from 72 h onward. In addition, F+C+ and F-C+ chickens received the antibiotic Colistin® (Polymyxin E; Dopharma B.V., Raamsdonksveer, the Netherlands) in their drinking water (200 mg Colistin sulphate /L water) until 21 days of age, whereas F+C- and F-C- chickens received drinking water without Colistin.

Exactly 72 hours after removal from the incubator, each chick was weighed again and 8 chickens per treatment group (spread over hatching times) were collected for autopsy. Autopsy chickens were decapitated and yolk free body mass (YFBM),residual yolk (RY) weight, heart weight, liver weight, and bursa

weight was determined.
The remaining chickens (56 per treatment) were divided over 32 floor pens (8 pens per treatment) in two different climate rooms. Treatments were equally distributed over each climate room. Seven chickens of the same treatment were housed per pen with a density of 4 birds/m^2. Body weight (individual) and feed intake (per pen) were determined weekly.
Housing temperature was 33°C at day 1 and gradually decreased until 20°C at day 35 until the end of the experiment (d 48; wk 7). Light/darkness program was as follows: wk 1-2 16:8, wk 3 14:10, wk 4 12:12, wk 5 11:13, wk 6 10:14 and wk 7 9:15.

Table 1. Body- and organ weights of 72 h old layer chickens that were fed immediately after hatch (F+) or 72 h later (F-), and were treated with the antibiotic Colistin for 72 h (C+) or were not treated (C-). Data represent least square means and s.e.m.

	Treatment					P-value		
Item[1]	F+C+	F+C-	F-C+	F-C-	s.e.m.	F	C	F x C
Body weight (g)	43.7	42.7	33.6	34.4	1.0	***	n.s.	n.s.
YFBM (g)	42.2	40.9	32.1	32.8	0.95	***	n.s.	n.s.
RY (g)	1.5	1.8	1.5	1.6	0.16	n.s.	n.s.	n.s.
Heart (% YFBM)	0.82	0.85	0.79	0.70	0.03	**	n.s.	#
Liver (% YFBM)	4.54	4.43	2.66	2.73	0.15	***	n.s.	n.s.
Bursa (% YFBM)	0.14	0.12	0.11	0.11	0.009	*	n.s.	n.s.

[1] YFBM = yolk free body mass; RY = residual yolk
0.05<*P*<0.10; *P<0.05; **P<0.01; ***P<0.001; n.s. = not significant

Eimeria infection
At d 27 of age, all chickens were inoculated in the crop with a phosphate buffered saline (PBS) solution containing 25,000 sporulated *Eimeria acervulina* oocysts (Animal Health Service, Deventer, the Netherlands). The dose of oocysts was based on a previous experiment of Walstra et al. (2010). At d 3, 4, and 7 post inoculation (p.i.), 8 chickens per treatment were euthanized by cervical dislocation, dissected, and the duodenum was visually scored for lesions. The severity of the lesions ranged from 0 (no lesions) to 4 (severe lesions) (Walstra et al., 2010). A faecal sample (± 2.0 g) was also collected from the colon of each dissected chick at d 4 p.i. and d 7 p.i. and the number of oocysts secreted per gram faeces (OPG) was counted with the salt floatation method of Long and Rowell (1975). BW of chickens was determined at the day of infection (d 0 p.i.), at d 3 p.i., d 7 p.i., and d 14 p.i. and feed intake was determined from d 0-3 p.i., from d 3-7 p.i. and from d 7-14 p.i.
Blood samples were taken from the wing vein with heparinized syringes of the same four chickens per pen at d 0, 3, 7, 14 and 21 p.i.. Blood was centrifuged at 4,750 rpm for 6 min to obtain plasma which was subsequently stored at -20°C before analysis. Antibodies to *E. acervulina* were quantified by ELISA (Graat et al., 1996).

Statistical analysis

For statistical analysis we used SAS software (SAS Institute Inc., Cary, NC, USA). Differences in body weight, YFBM, RY weight, heart weight, liver weight and bursa weight at 72 h after hatch were analysed with a PROC GLM for the effects of feed, Colistin and their interaction. The individual chicken was the experimental unit. Body weight and feed intake from hatch until the end of the experiment, as well as *Eimeria* antibody titre, were analysed with a PROC MIXED for repeated measurements for the effects of feed, Colistin, day and their interaction, with the pen as the experimental unit. BW gain after *E. acervulina* infection was analysed per period with a PROC GLM for the effect of feed, Colistin and their interaction. OPG after *E. acervulina* infection was analysed per day with a PROC GLM for the effects of feed, Colistin and their interaction with chick as experimental unit. Duodenal lesions were analysed per day with a PROC LOGISTIC test for the effects of feed, Colistin and their interaction. Chick was the experimental unit. Data are presented as least square means ± s.e.m. in tables and figures, unless stated otherwise. Non-significant interactions were excluded from analysis by stepwise deletion, except for the interaction between feed and Colistin treatment. OPG and antibody titre were not normally distributed and a log transformation was therefore performed before analysis. Effects were considered significant at $P \leq 0.05$ and a trend at $0.05 < P < 0.10$.

RESULTS

Body- and organ weight

There was no interaction between early feeding and Colistin treatment on body- and organ weight in the first 72 h post hatch ($P>0.05$). Feed provision in the first 72 h after hatch resulted in chickens with a higher body weight, YFBM, heart weight, liver weight, and bursa weight (Table 1; $P<0.05$), but no differences were observed in RY weight between early fed and non-fed chickens. Colistin treatment had no effect on body and organ weight at 72 h after hatch.

No interaction was found between early feeding and Colistin treatment for body weight, feed intake and feed conversion rate (FCR) throughout the experimental period ($P>0.05$). Early fed chickens had an overall higher feed intake ($P<0.001$; Figure 1b) and BW ($P<0.001$; Figure 1a) from hatch until the end of the experiment (d 48) than non-fed chickens and weighed approximately 47 g more at d 48 of age. Colistin treatment for 21 d had no effect on BW development and feed intake. No differences in overall FCR between F+C+ (2.49 ± 1.02), F+C- (2.57 ± 1.02), F-C+ (2.45 ± 1.02) and F-C- (2.54 ± 1.02) were found.

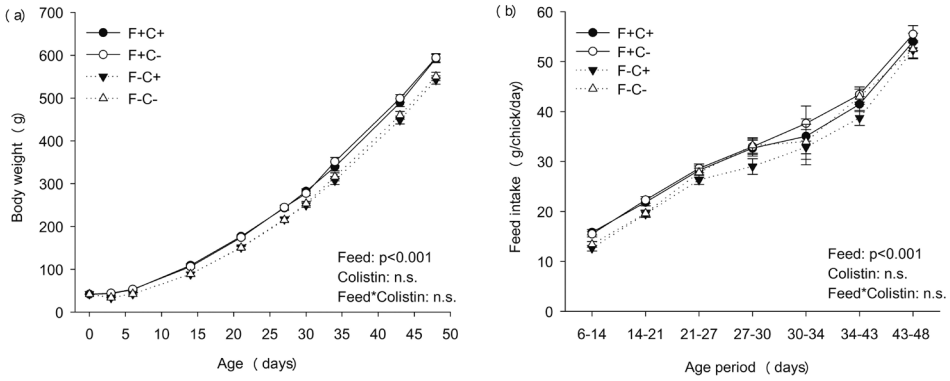

Figure 1. Body weight (a) and feed intake (b) of layer chickens that were fed immediately after hatch (F+) or non-fed for 72 h (F-), and were treated with the antibiotic Colistin for 21 d (C+) or were not treated (C-). Data represent least square means and s.e.m.

BW and feed intake after E. acervulina.

From d 0-3 p.i. (Figure 2a), an interaction between early feeding and Colistin treatment was found. Colistin treatment resulted in a lower BW gain of non-fed chickens, whereas it did not influence the BW gain of early fed chickens (P<0.001). Feed intake and FCR in the same period did not differ among treatments (Figure 1b). From d 3-7 p.i. (Figure 2b) there was no interaction between early feeding and Colistin treatment for BW gain and feed intake. Early fed chickens had a lower BW gain than non-fed chickens (P=0.005), but a higher feed intake (P=0.02; Figure 1). Moreover, Colistin treated chickens tended to gain less weight from d 3-7 p.i. than non-treated chickens (P=0.07) but no differences in feed intake and FCR were found (Figure 1b). There was no interaction between early feeding and Colistin treatment for BW gain and feed intake from d 7-16 p.i. Early fed chickens tended to have a lower BW gain than non-fed chickens (P=0.07). Colistin treated chickens had a lower feed intake (Figure 1b; P=0.04) and FCR (2.51 ± 0.04 vs. 2.69 ± 0.05) than non-treated chickens, but did not differ in BW gain. From the moment of infection until d 16 p.i., there was a tendency toward an interaction between early feeding and Colistin treatment for BW gain. Colistin treatment tended to reduce the BW gain of non-fed chickens, whereas it did not influence the BW gain of early fed chickens (P=0.08, Figure 2d). Moreover, early fed chickens had a significant lower BW gain in this period than non-fed chickens (P=0.001). No differences in feed intake (Figure 1b) and FCR were found among treatments.

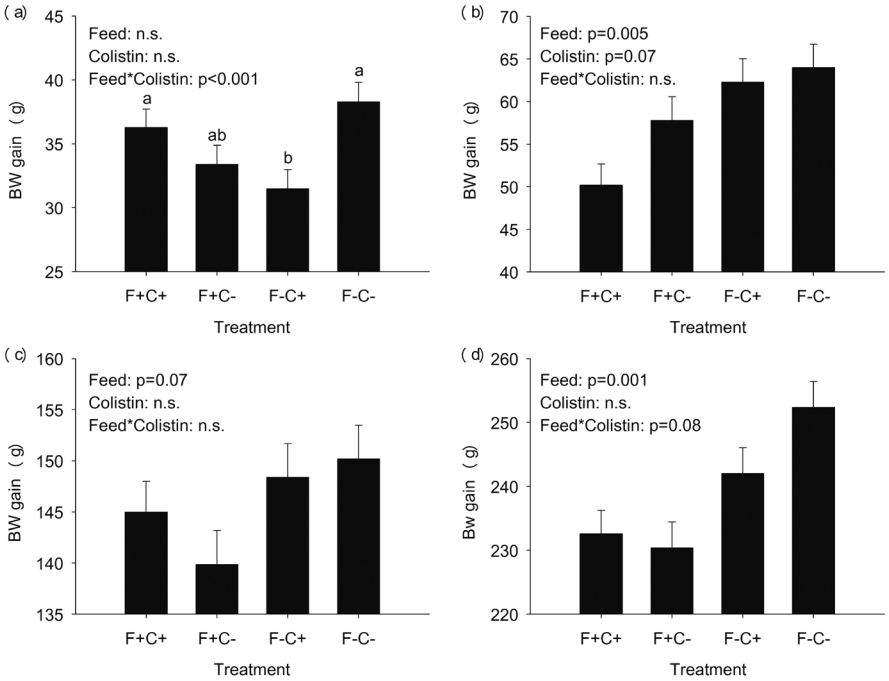

Figure 2. BW gain per chicken from d 0-3 (a), d 3-7 (b) and d 7-16 (c) and d 0-16 (d) after *E. acervulina* inoculation in 27-day old layer chickens that were fed immediately after hatch (F+) or 72 h later (F-), and were treated with the antibiotic Colistin for 21 d (C+) or were not treated (C-). Data represent least square means and s.e.m. Bars marked with different letters represent significant differences between treatment groups ($P<0.05$)

Table 2. Oocyst production per gram faeces (x104) at d 4 and d 7 after *E. acervulina* inoculation (25.000 oocyst/mL PBS) of layer chickens that were fed immediately after hatch (F+) or 72 h later (F-), and were treated with the antibiotic Colistin for 21 d (C+) or were not treated (C-). Data represent least square means and s.e.m.

	Treatment					P-value		
Time	F+C+	F+C-	F-C+	F-C-	s.e.m.	F	C	F x C
Day 4 p.i.	1.30	1.79	0.75	1.35	0.61	n.s.	n.s.	n.s.
Day 7 p.i.	42.87	64.95	24.87	25.14	15.04	*	n.s.	n.s.

*$P<0.05$; n.s. = not significant

Duodenal lesions and OPG

Dissection of the intestines at d 3, 4, and 7 p.i. demonstrated intestinal lesions in the duodenum due to *E. acervulina* (Figure 3). At d 3 p.i. (Figure 3a) and d 4 p.i. (Figure 3b) no differences in lesion severity among treatments were observed. If chickens were non-fed in the first 72 h post hatch, additional Colistin treatment from 0-21 d tended to reduce the degree of duodenal damage at d 7 p.i. (feed*Colistin interaction; *P*=0.06; Figure 3c). Moreover, early feeding tended to reduce the duodenal lesions at d 7 p.i. (*P*=0.10). The number of oocysts produced per gram faeces did not differ between treatments at d 4 p.i., but early fed chickens had a higher OPG than non-fed chickens at d 7 p.i. (Table 2; *P*=0.04).

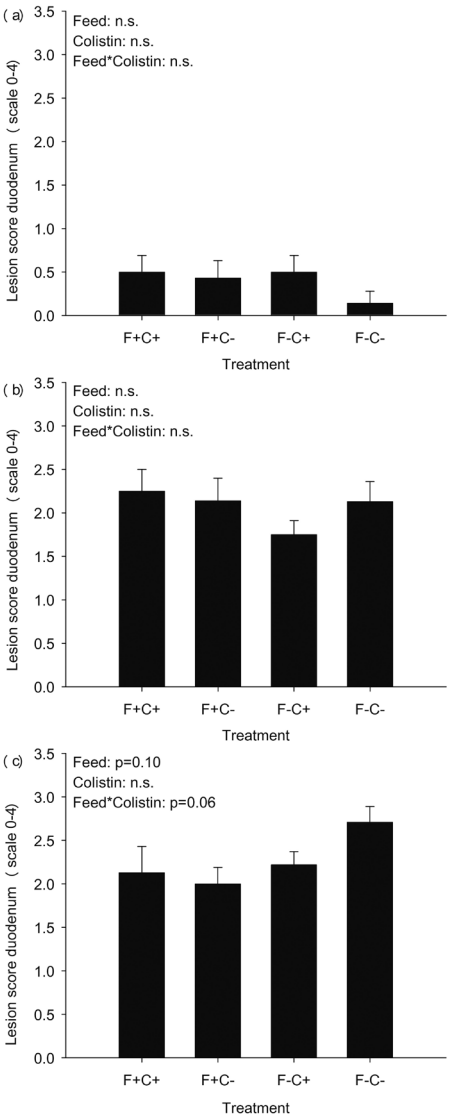

Figure 3. Lesion score in duodenum due to *E. acervulina* infection at d 3 (a), d 4 (b) and d 7 post inoculation (c) in layer chickens that were fed immediately after hatch (F+) or 72 h later (F-), and were treated with the antibiotic Colistin for 21 d (C+) or were not treated (C-). The score of the lesions ranged from 0 until 4 in which score 0 = no lesions; score 1 = 1 to maximum 5 lesions per cm2; score 2 = more than 5 separate lesions per cm2; score 3 = some lesions are merged but separate lesions are still visible; score 4 = lesions are merged and hardly visible individually (Animal Health Service, Deventer, the Netherlands). Data represent least square means and s.e.m.

Antibody responses to Eimeria acervulina

No interaction between early feeding and Colistin treatment was found in total antibody titre to *E. acervulina* (Figure 4). Total antibody titre was higher in early fed chickens than in non-fed chickens (P<0.001), and Colistin treated chickens had a lower antibody titre than non-treated chickens (P=0.01).

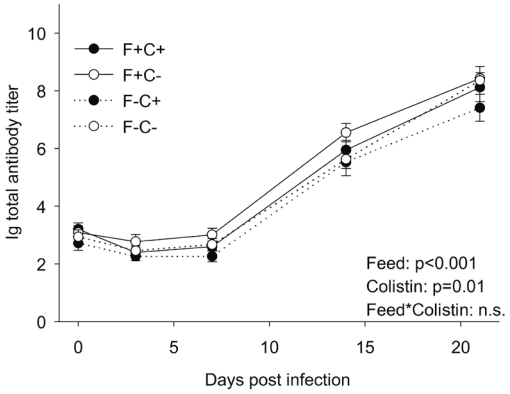

Figure 4. *E. acervulina* specific Ig total antibody responses after infection, of layer chickens that were fed immediately after hatch (F+) or 72 h later (F-), and were treated with the antibiotic Colistin for 21 d (C+) or were not treated (C). Data represent least square means and s.e.m.

DISCUSSION

The present study demonstrated that the provision of feed in the first 72 h post hatch had profound effects on organ and body growth of layer chickens. At 72 h post hatch, organ and body weight of early fed layers was higher than in non-fed layers, which corresponds to the results previously found in broilers (Dibner et al., 1998; Uni et al., 1998; Bar-Shira et al., 2005). Walstra et al., (accompanying paper 2011a) demonstrated that this body weight difference may even be maintained until d 76 of age. Thus, early feeding seems to have a positive influence on organ and body weight of young laying hens. The use of Colistin, however, had no effect on organ and body weight, which indicates that a reduction in the number of colonizing gram negative bacteria is not important for organ and body growth of layers.

After the infectious challenge with *Eimeria acervulina*, the adaptive response of the layers was measured by BW gain and feed intake at different moments after infection, both in the acute phase (d 0-3 and d 3-7 p.i.) and in the recovery phase (d 7-16 p.i.). Moreover, duodenal lesions, oocyst production per gram faeces (OPG) and systemic *Eimeria* antibody titre after primary infection were determined to gain insight in the response to the infection. Previous studies already demonstrated that measuring the above mentioned factors after chickens are inoculated with an equal amount of oocysts reflect the resistance or susceptible status of the individual (Lillehoj and Ruff, 1987; Lillehoj, 1988; Caron et al., 1997). Infection with *Eimeria acervulina* is characterized by reduced body-weight gain of the host (Turk, 1978) and we indeed found a small reduction in BW gain, however none of the layers lost weight after the infection. Moreover, BW gain after infection differed between the four treatments, and varied dependent on the period after the *Eimeria* challenge. Early fed layers were heavier and remained heavier than non-fed layers, but had a lower

BW gain between d 3-7 p.i., d 7-16 p.i. and over the entire period of d 0-16 p.i. Walstra et al. (accompanying paper 2011a) demonstrated earlier that early feeding for 72 h results in alterations in microbiota composition in the ileum and cecum, and it can easily be envisaged that early feeding may also influence the microbiota composition in the duodenum, which is the replication site of *Eimeria acervulina*. Especially because the gut associated lymphoid system is a complex system and changes in microbiota composition in one part of the intestines may affect the function of another part of the intestines (Brandtzaeg et al., 2008).

Because *Eimeria* is an intestinal parasite, it has to compete for adhesion sites with the already present intestinal bacteria (Dalloul et al., 2003). We hypothesize that it may therefore depend on the microbiota composition on how well the *Eimeria* parasite is able to penetrate the epithelial surfaces of the intestines. Easier penetration of the epithelial surface due to a less competitive intestinal microbiota composition will lead to more replication and shedding of oocysts (Dalloul et al., 2003). This is consistent with the OPG of early fed layers on d 7 p.i., which is higher than the OPG of non-fed layers. The higher antibody titre in early fed layers as compared to non-fed chickens would also fit this explanation, as more replication of *Eimeria* oocysts will result in a higher antigen load and therefore a higher antibody production as response. Although early feeding is known to stimulate GALT development and thereby has a positive influence on the immune response of young chickens (Dibner et al., 1998; Bar-Shira et al., 2005), the results in the present study seem to suggest that the response to *Eimeria acervulina* is not positively affected, as early fed chickens gain less weight, and have a higher OPG and antibody titre than non-fed chickens. On the other hand, early fed layers tended to have lower lesion scores at d 7 p.i., which normally indicates intestinal invasion of the parasite and would suggest that although there is more replication in early fed chickens, they seem to recover faster.

Therefore, another suggestion is that early fed layers rely on a different adaptive strategy. Allowing their body mass to reduce more, while increasing their antibody titre and oocyst shedding may well be an adaptive strategy to combat the infection, that is more efficient in getting rid of the pathogen (Hart, 1988). Animals often have an adaptive strategy in response to an infection (i.e. sickness response). The observation that duodenal lesions are lower at d 7 p.i., indicating a faster recovery, is in favour of this suggestion.

Modulation of the (gram negative) intestinal microbiota by using Colistin had hardly any effect on BW gain after the *Eimeria* infection. An interaction was observed both in de acute phase of the *Eimeria* infection (d 0-3 p.i.) and over the complete period p.i. (d 0-16). Colistin treatment only had an additional effect on the BW gain of non-fed chickens, whereas it did not influence the BW gain of fed chickens. Previous studies (Van Saene et al., 1988; Walstra et al. accompanying paper 2011a) demonstrated that Colistin treatment affected the microbiota composition of the intestines. Early fed layers are probably exposed to a wide variety of bacteria after ingestion of their feed and it is possible that treatment with Colistin and the inhibition of gram negative bacterial colonization has less effects in these layers. However, this remains a suggestion. We did observe differences in *Eimeria* antibody titre, which was lower in Colistin treated layers as compared to non-treated layers. This does indicate a role for early colonization of gram negative bacteria in the intestines in the later life humoral immune response during an *Eimeria* infection. This confirms our hypothesis

previously stated in Walstra et al. (accompanying paper 2011a), that the microbiota composition may particularly influence the adaptive immune responses in the period that the immune system of the chicken is still developing.
From the results in the present study we can conclude that early feeding is positive for growth, but also that both early feeding and Colistin treatment result in changed adaptive responses to an *Eimeria acervulina* infection, indicating that the microbiota composition in early life may play a role in the adaptive capacity of layers.

ACKNOWLEDGMENTS

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GENERAL DISCUSSION

INTRODUCTION

In commercial husbandry systems, laying hens may encounter numerous environmental challenges, including the exposure to different pathogens. The current strategy to deal with pathogens in the animal production industry is based on prevention and control methods (control model: Ten Napel et al., 2006), and primarily aimed at minimizing the risk of infection with the pathogen. Biosecurity measures, hygiene practices, vaccination programs, feed additives and treatment or culling of infected or susceptible flocks are all part of the control strategy and are used worldwide. The use of antibiotics as part of the control strategy is the subject of an on-going debate in the intensive animal production sector, as it poses risks for both animal and human health, because it is linked to the emergence of antibiotic-resistant bacterial strains (Hughes et al., 2008). This requires a search for alternatives to improve animal health and disease resistance. This thesis therefore focused on a possible alternative strategy for animal health and disease control, by utilizing the intrinsic adaptive capacity of layers for maintaining their own health and welfare during (infectious) challenges. Layers were mainly chosen as model animals to influence the adaptive capacity, as both the pre- and postnatal environmental conditions of layers can easily be influenced without maternal interference, however the results of this thesis may also apply to other species in animal production. The focus of this thesis was mainly on the ability to adapt to infectious challenges, in which a good adaptability would mean that the layer has minimal clinical symptoms and a fast recovery after infection.

Moreover, this thesis focused mainly on the adaptive capacity of layers in the rearing period. The rearing period is an important period as it prepares the layers for the production period, in which they are expected to be well developed, healthy and lay as many as 300 eggs a year. It is therefore important that layers develop well in the rearing period and are already able to withstand any challenges they will encounter in the husbandry systems. The experiments in this thesis applied contrasts in early life environmental conditions as they may occur in commercial husbandry systems for layers. Important environmental conditions in early life that were explored in this thesis were incubation temperature, early feed and water availability post hatch, manipulation of early intestinal microbiota composition with the antibiotic Colistin, housing temperature in the first 10 days post hatch, and enrichment of the rearing environment. The experiments aimed at challenging the adaptive capacity of the layers with infectious challenges. The adaptive capacity of the layers was determined by measuring the responses to these challenges, either behavioural, physiological or immunological.

In this discussion, the possibilities to influence the adaptive capacity of layers during infectious challenges, with early life conditions as they may occur in practice, are discussed. Moreover, this discussion focuses on possible explanations for the effects of early life conditions on the later life adaptive capacity of layers during infectious challenges. Finally, the conclusions and implications of the results in the current thesis are presented.

STANDARD PRACTICE VERSUS OPTIMIZED EARLY LIFE CONDITIONS

It was hypothesized that the adaptive capacity of young layers later in the rearing period could be influenced by applying contrasts within the current layer hatchery and rearing practice. These different contrasts will first be discussed in this section.

1. One contrast applied was a contrast in incubation temperature. In current practice, incubation occurs in large single- or multi stage incubators with a capacity of up to tens of thousands of eggs. In these modern incubators and also during incubation trails (French 1997), the machine temperature is usually the treatment applied to the eggs and will be set around 37.5°C (Lourens, 2001). However, the machine temperature is different from the embryo temperature, due to the imbalance between embryonic heat production and (latent) heat transfer in early and late incubation, and location of the egg in the incubator (French, 1997; Lourens, 2001; Hulet et al., 2007; Chapter 2, results not shown). The embryo temperature can be closely estimated by the eggshell temperature (EST; Lourens, 2001). It was shown by Lourens (2001) that with a constant machine temperature the EST within a machine varied between 36.2 and 40.2°C, depending on the place of the egg in the incubator and the day of incubation. Commercial incubators often use a step-down machine temperature program to prevent overheating of the embryo at the last stage of incubation, when embryonic heat production is highest. It was previously demonstrated in broilers that a commercial single stage incubator set at a step-down machine temperature program of 38.0°C at embryonic day (ED) 1 to 37.2°C at ED 18, resulted in an average EST close to 37.8oC, however, depending on the position in the incubator, EST ranged between 36.2 - 37.8°C at ED 1, and between 37.8 - 40.2°C at ED 18 (Lourens, 2001). Because incubation temperature is one of the most important factors of embryonic development, these deviations may have a large impact on embryonic development, hatchability and post hatch performance (Deeming and Ferguson, 1991; Decuypere and Michels, 1992; Lourens et al., 2005). Based on these previous studies, the first contrast applied was suboptimal versus optimized EST. In the suboptimal EST, a low EST (36.7°C) in the first week, a normal/optimal EST in the second week (37.8°C) and a high EST (38.9°C) in the last week of incubation was applied to mimic standard conditions in practice, whereas an EST of 37.8°C throughout incubation was used in the optimized treatment (Chapter 2, 4). Another incubation temperature treatment applied in this thesis was thermal manipulation with periods of high EST in late incubation vs. normal incubation (Chapter 3). Thermal manipulation is a method to increase postnatal adaptability to high or low environmental temperatures, by exposing embryos to high or low temperatures during critical developmental periods in incubation (Tzschentke et al., 2004; Yahav, 2009). This is also referred to as epigenetic temperature adaptation (Nichelmann, 1992) and it is based on the knowledge that during embryonic development, temperature can influence the development and maturation of physiological control systems that are important for adaptation to high or low environmental temperatures post hatch and later in life, such as the thermoregulatory system and HPA-axis (Yahav et al., 2009). Previous studies on thermal manipulation only looked at meat producing breeds, such as broilers and ducks (Piestun et al., 2008, 2009; Yahav et al., 2004 a,b) but the possibilities to use TM in layers were never investigated. It may, however, also be important for laying hens, and not only because TM can influence the adaptive capacity during high temperatures post hatch. It was hypothesized that increasing the general ability of layers to cope with high ambient temperatures may be a method to make them less susceptible to pathogens that occur during these high temperature conditions. This is

important because high ambient temperatures are common in commercial husbandry systems nowadays, as more and more housing systems have an outdoor range. Previous observations demonstrated that high environmental temperatures resulted in physiological and immunological adaptations in the animal (Mashaly et al., 2004, Benderlioglu et al., 2007). Applying thermal manipulation may therefore be used as an indirect way to influence the adaptive capacity to infectious challenges of layers in commercial husbandry systems. Therefore, a pilot study was conducted to investigate the possibilities to use TM for layers (Chapter 3). However, the results in Chapter 3 did not demonstrate long lasting effects of thermal manipulation in layers. It is possible that the thermoregulatory adaptive response of layers was not challenged enough in this study, because the temperature during the thermal challenge was not high enough. When investigating thermal tolerance, a strong hyperthermia is needed to demonstrate any differences between the treatments applied (Yahav et al., 2009). Any individual variation in thermoregulatory adaptation capacity goes unnoticed if the challenge is too mild. The results in Chapter 3 demonstrated that the T_b of thermally challenged chicks increased with only 0.3°C at d 34, which cannot be referred to as a hyperthermic response. Furthermore, the timing and duration is important in order for TM to be effective and the period between ED 14 and ED 18 used in the experiment in Chapter 3 may just not be the right timing (Yahav et al., 2009). However, because the focus of this thesis was not on thermal manipulation but improving the adaptive capacity to infectious challenges, it was decided that the TM protocol would not be further used in this thesis. Instead, the effects of incubation temperature on the adaptive capacity were investigated by comparing suboptimal and optimal EST.

2. The second contrast applied within current practice conditions was a contrast in hatching environment. After 19-21 days of incubation, chickens hatch in the incubator where the temperature is around 37.5°C until pull out, and dependent on the time of hatching, hatchery protocols, transport etc., it may take up to 24-72 hours until the hatched chickens are placed in the rearing environment where they receive their first feed and water. Due to the high ambient temperature and lack of drinking water in the incubator after hatching, the hatchlings are likely to experience heat stress and dehydration, and a delayed access to feed is known to influence growth and (intestinal) development (Dibner et al., 1998; Bar-Shira et al., 2005). Therefore, the contrasts in EST in Chapter 2 were combined with contrasts in hatching conditions, with standard hatch as described above for the suboptimal treatment and optimized hatch in combination with the optimized incubation treatment. In the optimized hatch treatment, dry chickens (6 h old) were removed from the incubator and housed at a lower temperature (34°C) with immediate access to feed, water and foraging material. Moreover, during the first 10 days post hatching, these chickens received point heating in their pen, to provide the opportunity to maintain their optimal body temperature. In Chapter 5 and 6, hatching conditions (e.g. temperature, foraging materials etc.) were kept equal and a contrast in early feeding vs. feed deprivation in the first 72 h post hatching was applied. Furthermore, because effects of early feeding were suggested to be related to differences in microbiota colonization of the intestines (Bar-Shira et

al., 2005), a contrast was applied in which gram negative microbial colonization was reduced by the use of the antibiotic Colistin.

3. Rearing conditions for layers in practice range from cage systems (banned from the European Union in 2012), to aviaries, floor and free-range systems. They differ in space and possibilities for the layer to show natural behaviour, such as dust bathing, nesting and foraging. Therefore, the last contrast applied was a contrast in rearing conditions from hatching until week 7 of age (Chapter 2). This was done by using a cage (barren) or floor system (enriched with wood shavings, peat dust and perch). This contrast was applied immediately after suboptimal vs. optimal incubation plus hatch. After 7 weeks of age, contrasts in rearing environment ended and all layers were housed in the floor system.

After applying the early life contrasts, layers were not immediately exposed to the infectious challenges. This was done to distinguish present conditions from experience and ensure that the effects of early life conditions on the adaptive capacity were not just a direct effect but a carry-over effect.

EFFECTS OF EARLY LIFE CONDITIONS PRE- AND POST-CHALLENGE

The development of the adaptive capacity of animals was known to be influenced by early life conditions and experiences (Star, 2008) and the results in the current thesis demonstrated that the adaptive capacity later in life was affected by early life conditions as they may occur in commercial husbandry systems for layers. It was demonstrated that contrasts in early life conditions lead to layers that behave differently, i.e. both in performance pre- and post-challenge, but also in behavioural and immune responses. This occurred not only when contrasts were large and combined, as described in Chapter 2, but also when contrasts were separated and only focused on incubation or hatching conditions, as in Chapters 4-6. Moreover, the changed adaptive capacity was not only observed in one particular disease model, but in all infectious and non-infectious models used (*Eimeria* parasite, IB virus and LPS/HuSA challenge).

Pre- challenge performance parameters

The contrasts in early life conditions chosen in this thesis were considered optimal versus suboptimal for growth and development of the layers, and it was hypothesized that optimized conditions would result in a head start in development and would subsequently contribute to a better adaptive capacity. This was mainly based on previous studies on effects of incubation temperature on post hatch performance (Lourens et al., 2005, 2007; Joseph et al., 2006, Piestun et al., 2009), early feeding and post hatch performance (Dibner et al., 1998; Bar-Shira et al., 2005) and enrichment of the rearing environment (Jones, 1982; Gvaryahu et al., 1994; Cornetto et al., 2002). These results were confirmed by findings in Chapters 2, 4, 5 and 6 in the current|thesis. For pre-challenge parameters the experiments in the current thesis investigated effects on body and organ weight, chick quality at hatching, feed intake, behaviour and endocrinological parameters in the rearing period.

Chick length at hatching was longer after incubating at optimal incubation temperature (37.8°C) compared to suboptimal incubation temperature (Molenaar et al., 2010b; Chapter 2, trend; Chapter 4) and optimal incubation temperature also resulted in a better navel condition (Chapter 4). Both a longer chick length

and a better navel condition are indicators for increased chick quality (Wolanski et al., 2004; Fasenko and O'Dea 2008; Molenaar et al., 2008). Optimal incubation temperature did not influence body development and growth of layers at hatching and onward compared to suboptimal incubation temperature. No differences in yolk free body mass (YFBM) were observed at hatching and no differences in BW and feed intake in the rearing period (Chapter 4). However, optimal incubation temperature resulted in a higher heart weights at hatching (0.58% BW vs. 0.49% BW), which was previously shown in studies with high EST in late incubation (Molenaar et al., 2010a). Molenaar et al. (2010a) also demonstrated that optimal vs. suboptimal incubation temperature (= high EST in late incubation) resulted in differences in other organ weights, such as liver, spleen, bursa and intestines. In summary, suboptimal incubation temperature resulted in differences in chick quality and heart weight, but no differences in development, body weight and feed intake were observed in the rearing period until the moment of the infectious challenge.

Early feeding for 72 h resulted in layers with an increased body and organ weight (Chapter 5, 6). Early fed layers had a higher YFBM (Chapter 5) at 72 h post hatching and subsequently a higher body weight that remained higher until d 76 of age (Chapter 5). This observation confirmed previous studies in broilers where feed was provided immediately after hatching (Dibner et al., 1998, Uni et al., 1998, Bar Shira et al., 2005). This was probably mainly caused by a higher feed intake in early fed layers from hatching until day 43 of age. This higher feed intake also increased growth of the organs in the first 72 h, as was shown in Chapter 6. Early feeding for 72 h resulted in significantly higher heart, liver and bursa weights relative to total weight (Chapter 6). Moreover, early feeding influenced the microbial composition in both the caecum and the ileum, as was also the result of treatment with the antibiotic Colistin for 21 d (Chapter 5). Treatment with Colistin, however, did not influence any of the performance parameters discussed above. These results demonstrated, that early fed and non-fed layers differed both in growth and intestinal microbiota composition at the moment of the infectious challenges.

Both changes in organ weights and microbial composition of the intestines due to early feeding and/or Colistin treatment may be important for the adaptive response of layers later in life when they are exposed to an infectious challenge. The colonization of the gut with bacterial populations starts immediately after hatching when the chick starts foraging. As previously suggested by Bar-Shira et al. (2005), non-fed layers probably have a slower development of bacterial populations in the caecum, because they are not exposed to feed (which contains many bacteria) immediately after hatching. Therefore, the development of the gut associated lymphoid tissue (GALT) is also delayed, which may subsequently affect the local and systemic immune responses in later life.

Moreover, the bursa of Fabricius, which was heavier in early fed layers (Chapter 6) is a lymphoid organ that plays a role in antibody production in the chicken (Dibner et al., 1998). Therefore, factors that influence bursa development, such as early feeding (Dibner et al., 1998; Chapter 6), but possibly also incubation temperature (Molenaar et al. 2010a; Oznurlu et al., 2010) may (indirectly) influence the post hatching immune response. However, because this was not directly investigated in the current thesis, it remains a hypothesis that requires further investigation.

Apart from effects on performance, the contrasts in early life conditions also resulted in differences in tonic immobility behaviour (Chapter 2) and corticostero-

ne levels after a manual restraint stressor (Chapter 4). Optimized incubation plus hatching resulted in a reduction in tonic immobility duration compared with suboptimal incubation plus hatching, which is an indication for reduced fearfulness in optimized layers (Jones et al., 1994; (Chapter 2). Moreover, optimized incubation as a single early life condition resulted in higher corticosterone levels after manual restraint (Chapter 4). This suggests that the early life conditions the layers experience, in this particular case mainly related to the incubation environment, may have an effect on the functioning and responsiveness of the HPA-axis, which was previously demonstrated by Arjona et al. (1988). From ED 14 onwards, functional development and maturation of the HPA-axis occurs (Jenkins and Porter, 2004) and environmental factors, such as temperature, may influence this process and thereby the eventual set-point of the HPA-axis post hatch and in later life. When the HPA-axis is fully functional post hatch, the secreted adrenocortical steroids have a significant influence on functions that are important and critical for the layer's growth, post hatch survival, and performance, including the function of the immune system (Jenkins and Porter, 2004; Silverman et al., 2005). An altered responsiveness of the HPA axis post hatch due to suboptimal incubation temperature may therefore result in altered functioning of the immune system in times of an infectious challenge and could possibly influence the disease susceptibility or resistance via this pathway.
In summary, the early life conditions that were applied in the experiments in this thesis and that were based on optimized versus more standard early life conditions in practice, indeed have the ability to influence chick quality, organ weights, growth, feed intake and behaviour of layers post hatching and in later life.

Post challenge performance and immune parameters
The model infectious challenges in the current thesis were used to challenge the adaptive capacity of layers subjected to different early life conditions. The experiments described in this thesis demonstrated that different early life conditions resulted in altered responses to both infectious (*Eimeria* parasite, IB virus) and non-infectious (LPS/HuSA) challenges. This was measured by differences in performance response, i.e. body weight (gain) and feed intake, but also factors describing disease resistance (duodenal lesions and oocyst production after *Eimeria*; Chapters 2, 4, 6) and by differences in immune responses, such as the area of T-cells and macrophages in the duodenum (Chapter 2), as well as different antibody responses (Chapter 2,5,6). Table 1 summarizes the effects of the early life conditions used in this thesis on both performance and immune responses after the challenges. The results of this Table will be discussed in the next paragraphs.

Infectious challenges; Eimeria and IB
The first model infectious challenge that was used to challenge the adaptive capacity after each applied early life condition, was the *Eimeria* parasite, responsible for the disease Coccidiosis. This allows us to compare the adaptability to *Eimeria* between the different early life conditions. Weight gain after inoculation with *Eimeria* oocysts is an important clinical sign of the severity of the infection and a reduction in BW gain or a loss of body weight is a distinctive reaction to the infection (Turk, 1978). The results in this thesis showed that BW gain after *Eimeria* inoculation was less reduced when contrasts in incubation, hatching and rearing conditions were optimized, and these 'optimal-enriched' layers had the highest feed intake

Table 1. Summarized effects of differences ($P<0.05$) in adaptive response to disease challenges. Parameters were either increased (↑), decreased (↓) not significantly affected (↔) or not measured (n.a.) when compared with the standard practice treatments, unless stated otherwise (interactions). Standard practice parameters were suboptimal EST + hatch (Chapter 2), cage rearing (Chapter 2), suboptimal EST (Chapter 4), feed deprived for 72 h (F-; Chapter 5,6) and no Colistin treatment (C-; Chapter 5,6).

	Optimal EST + hatch (OI)	Enriched rearing (E)	Optimal EST	Early feeding (F+)	Colistin (C+)
Parameter	Chapter 2 (*Eimeria* and IB)		Chapter 4 (*Eimeria*)	Chapter 5 (LPS/HuSA) and 6 (*Eimeria*)	
BW gain *Eimeria*	↑ (interaction: OI x E highest)		↑*	↓	↔
BW gain IB	↔	↑	n.a.	n.a.	n.a.
BW gain LPS/HuSA	n.a.	n.a.	n.a.	↓ (interaction: F+C- lowest d 0-1 p.i.)	
Feed intake *Eimeria*	↑ (interaction: OI x E highest)		↑*	↑	↔
Feed intake IB	↔	↔	n.a.	n.a.	n.a.
Eimeria lesions	↔	↓	↓ * (d 3 p.i.)	↓*(interaction: C+ reduced F- lesions)	
Eimeria OPG	n.a.	n.a.	↓ (d 7 p.i.)	↑ (d 7 p.i.)	↔
Antibody *Eimeria*	n.a.	n.a.	n.a.	↑	↓
Antibody IB	↔	↓	n.a.	n.a.	n.a.
Antibody LPS/HuSA	n.a.	n.a.	n.a.	↑ (interaction: F+C+ highest)	
Antibodies KLH (Nab)	n.a.	n.a.	n.a.	↑ (interaction: F+C+ highest)	
CD4+ T cells[1]	↔	↔	n.a.	n.a.	n.a.
CD8+ T cells[1]	↔	↔	n.a.	n.a.	n.a.
Macrophages[1]	↓	↔	n.a.	n.a.	n.a.

[1] = measured in duodenum

*= tendency (P<0.10)

and the lowest weight loss after the *Eimeria* challenge (Chapter 2). The same was observed during the IB infection, where feed intake and BW gain was also highest in layers in the optimal – enriched treatment.

Feed intake and BW gain after *Eimeria* also tended to be less reduced when optimal incubation temperature was applied as a single early life condition, however in this experiment layers did not lose weight (Chapter 4). This can be explained by differences in *Eimeria* species used. In Chapter 2, a mixed species infection was given with *E. acervulina*, *maxima* and *tenella*, whether in Chapter 4 only *E. acervulina* was used, which is less pathogenic than *maxima* and *tenella* and usually only a reduction in weight gain is observed after *E. acervulina* infections, and no weight loss (Turk, 1978).

The results in Chapter 6 showed that early feeding resulted in a higher average body weight, as discussed in the previous paragraph, but early fed layers had a lower body weight gain after inoculation with *Eimeria*, whereas feed intake remained higher in early fed than non-fed layers. This could be an indication that early fed layers are less efficient during infection, needing more feed to gain the same amount of weight as non-fed layers, however, no differences in FCR were observed

in Chapter 6 (results not shown).

These results demonstrated that optimal incubation temperature, whether or not combined with optimal hatching and enriched rearing, has positive effects on the weight gain of layers after a challenge with *Eimeria* and IB (Chapter 2, 4). In rearing farms, a higher weight gain during infectious diseases is usually considered better, and layers that maintain their weight gains are most likely to be considered having a higher adaptive capacity to the infection. However, does this mean that early fed layers have a lower capacity to adapt to the *Eimeria* challenge than non-fed layers, because they gain less weight? As was described in the previous section on pre-challenge performance parameters, early fed layers had a higher BW before and after the *Eimeria* challenge as compared to non-fed layers. They only gained less weight during the challenge. In order to gain more insight in whether the reduced weight gain should indeed be considered positive or negative for the adaptive capacity of the layers, we also have to consider other parameters that are indicative for the adaptive response to *Eimeria*, such as the oocyst production per gram faeces and duodenal lesions after inoculation.

The number of oocysts shed in the faeces after ingestion of a fixed amount of oocysts is considered a measure of disease resistance (Lillehoj and Ruff, 1987) and results in this thesis demonstrated that optimal incubation temperature resulted in a reduction in the number of oocysts shed at d 7 p.i. (Chapter 4). Moreover, a reduced number of intestinal lesions was observed in optimal incubated layers at d 3 p.i, indicating that optimal incubated layers had a less severe intestinal response to the infection.

The number of oocysts shed in early fed layers was higher at d 7 p.i. than the number of oocysts shed in non-fed layers at d 7 p.i. (Chapter 5). For duodenal lesions, non-fed layers had more lesions at d 7 p.i. than early fed layers, but only in combination with Colistin treatment for 21 d post hatch. Day 7 p.i. is already the start of the recovery phase of an *Eimeria* infection, and the severity of intestinal lesions at this day might give some indication about the ability of the layers to recover from the infection.

The differences observed in response to *Eimeria* and IB in layers exposed to different early life conditions are likely to be accompanied by differences in immune response. Results of some immunological parameters measured in this thesis demonstrated that there were significant effects on both cell mediated and humoral immune responses after *Eimeria* inoculation. Optimal incubation plus hatch resulted in a reduced area of macrophages in the duodenum after *Eimeria* challenge (Chapter 2). Macrophages are part of the innate arm of the immune system, which suggests that optimal incubation plus hatch affects the innate immune response. Unfortunately, in Chapters 2, 4 and 6 no other innate immune responses after *Eimeria* were measured to confirm these results.

Early life conditions applied in Chapter 2 also affected the adaptive humoral immune response to the IB virus. IB antibody response was mainly affected by the rearing environment in Chapter 2, and the IB titre of cage reared layers was higher and increased faster after IB infection compared to enriched reared layers. Because the IB challenge was given to the layers after prior vaccination against IB at d1 and d 15 of age, the height of the antibody response may also say something about the effectiveness of the vaccination, which took place during exposure to different rearing environments, rather than about the adaptive capacity of cage

and enriched reared layers to respond to the challenge. However, the titre response to IB vaccination was not different between cage and enriched reared layers (Figure 3, Chapter 2).
Early fed layers had a faster and higher antibody response to *Eimeria acervulina* than non-fed layers, whereas manipulation of the intestinal microbiota resulted in a lower antibody titre to *Eimeria acervulina* (Chapter 6). These results suggest that both early feeding and manipulation of intestinal gram negative microbiota with Colistin also influence the humoral adaptive immune response. It was considered that the ability to generate a fast and high antibody response to *Eimeria acervulina* is in favour of adaptation during the challenge, which indicates (based on the antibody levels) that early fed layers adapt better to the *Eimeria acervulina* challenge in later life.

No- infectious challenge; LPS/HuSA
A model antigen challenge with a mixture of LPS and HuSA was given to layers in the early feeding and Colistin treatments at d 62 and d 63 of age, as described in Chapter 5. HuSA was administered together with LPS, to investigate the effects of the early life conditions applied on an immune challenge that involved both a T-cell dependent (HuSA) and T-cell independent (LPS) activation of the humoral immune system.
In the first 24 h post challenge (d 0-1 p.i.), early fed layers that were not treated with Colistin lost approximately 12 g weight, whereas layers of all the other treatments only had a reduced weight gain, but did not lose weight. It may be possible that the intratracheal LPS/HuSA challenge was not successful in all treatments the first time (d 62), which is also the reason why the challenge was given a second time in all treatments at d 63 of age. However, from d 1-2 p.i., which was 0-24 h after the second challenge, no differences were observed in BW gain between treatments, which indicates that the first challenge was indeed successful and early fed layers that were not treated with Colistin were the only treatment that showed the LPS induced body weight loss. Previously it was shown that exposure to LPS induced the cachectin activities of acute phase proteins such as interleukin (IL)-1 and IL-6 or tumor necrosis factor (TNF) a- like substances. Cachectins induce a state of anorexia, in which the animal loses BW (Parmentier et al., 1998), which is all part of the sickness response as a strategy to adapt to the pathogen. From this point of view, it seems that early fed layers that were not exposed to Colistin have a better adaptive response after LPS/HuSA challenge (with regard to BW gain) than layers from the other treatments. However, this was not reflected in the immune responses. Early feeding and manipulation of the gram negative microbial composition with Colistin influenced the humoral immune system, as could be observed in both the specific (T-cell independent LPS-titre), and the natural antibody (NAB) titre to KLH, which is part of the innate immune response. However no effects were found on specific T-cell dependent HuSA titre. Both for LPS and KLH titre, early fed layers, and specifically early fed layers that were exposed to Colistin (F+C+), had a higher antibody titre for both LPS and KLH.
Although the LPS antibody titre in Chapter 5 is considered to be a specific (adaptive) response to LPS exposure, as layers were first challenged with LPS and subsequently the antibody response to LPS was determined, there was already a baseline titre difference between layers of the different treatments (Figure 6,

Chapter 5). This indicates that the innate immune response to LPS might be more important in this matter. When correcting for the baseline difference, no differences in LPS titre were observed anymore. This confirms our previous statement that the differences in LPS titre are actually more related to differences in natural antibodies, which is also the case for the KLH titre. Thus, both NAB binding to LPS and KLH is higher in F+C+ layers. Previous studies have already demonstrated a correlation between NAB binding to LPS and KLH, and the present study suggests the same (Minozzi et al., 2007). Moreover, these results also suggest that early feeding and Colistin treatment had no effects on the adaptive humoral immune response (Chapter 5).

When we compare the response of layers in Chapter 6 to the response of layers in Chapter 5, it seems that the adaptive immune system was affected in Colistin treated layers exposed to *Eimeria acervulina* (Chapter 6), but, not in Colistin treated layers exposed to LPS/HuSA (Chapter 5). It was hypothesized that this difference may be explained by the fact that the gram negative microbiota (which are reduced with Colistin) has an effect on the adaptive immune response during the period in which maturation of the immune system still occurs, which is from hatching until around nine weeks of age, but not anymore after the immune maturation process is complete. This means that when manipulation of gram negative microbiota is used as early life contrast, it may only have an effect on the adaptive immune response in very young layers.

However, it is also possible that no effects on the adaptive immune system were found because the period between the end of the Colistin treatment and the challenge with LPS/HuSA is too long. Because Colistin treated layers are also exposed to LPS, due to the presence of LPS in their environment (e.g. in dust and faeces) from the end of the Colistin contrast (d 21) until the LPS/HuSA challenge (d 62), immune reactivity will probably already recover and reach the same level as layers not treated with Colistin. This may be a statement that requires further investigation.

ADAPTIVE CAPACITY

The early life conditions applied in this thesis result in layers that differ in both performance and immune parameters after infectious challenges. The differences in body and organ weights, behaviour and endocrinology that were found before the infectious challenges, may be an underlying mechanism for the differences in adaptive response to the infectious challenges.

The infectious challenges used in this thesis differ from one another, *Eimeria* is a parasitic infection whereas IB is a virus infection and LPS/HuSA is a microbial non-infectious model challenge to mimic a lung infection. Clinical symptoms after these infections are different and the responses to the challenges are most likely regulated by different arms of the immune system. When interpreting the results of challenge experiments such as the experiments performed in the current thesis, it becomes apparent that animals may use different strategies to adapt. Therefore, looking at one particular response parameter, such as BW gain after challenge, may not be the best method to determine whether the adaptive capacity to the challenge is improved or not.

Thus the question arises, when do the layers have a better capacity to adapt? A high and fast immune response is usually associated with a better capacity of the immune system to adapt to the challenge and this was also the assumption

that was made at the start of the experiments. It was therefore hypothesized that alower response indicated a lower capacity of the immune system to respond to that particular challenge, which would be unfavourable for the adaptive capacity. However, a low antibody or cellular response to a challenge may also indicate that this particular animal does not need a high immune activation to respond to the challenge, which would then indeed be beneficial for the adaptive capacity. For example, the IB titre of enriched reared layers in Chapter 2 increased slower and was lower than the titre of cage reared layers. However, it was also demonstrated that BW gain after IB was less reduced by enriched rearing than by cage rearing. Based on these results we conclude that enriched reared layers may not need a high antibody response to the IB infection in order to keep functioning after the IB challenge and we therefore state that enriched rearing results in a better capacity to respond to the IB challenge. The same was observed for layers that received early feeding in Chapter 6. Early fed layers were heavier, but gained less weight after the *Eimeria* challenge, and shed more oocyst in their faeces, while they had a higher antibody titre in response to the challenge and lower lesions at d 7 p.i.

The results of this thesis indicate that layers may use different adaptive strategies to cope with the infectious challenges later in life. These adaptive strategies are dependent on the life history of the animal, but most likely also on the layer's current environment. When interpreting the influence of early life conditions on the adaptive capacity to infectious challenges later in life it is therefore better to consider the different read out parameters together (i.e. BW gain, feed intake, antibody levels, oocyst production etc.). This may give the best insight in whether the early life condition applied is positive or negative for the adaptability to the challenge. In general it seems that optimizing conditions in early life contributes in a positive manner to the adaptive capacity.

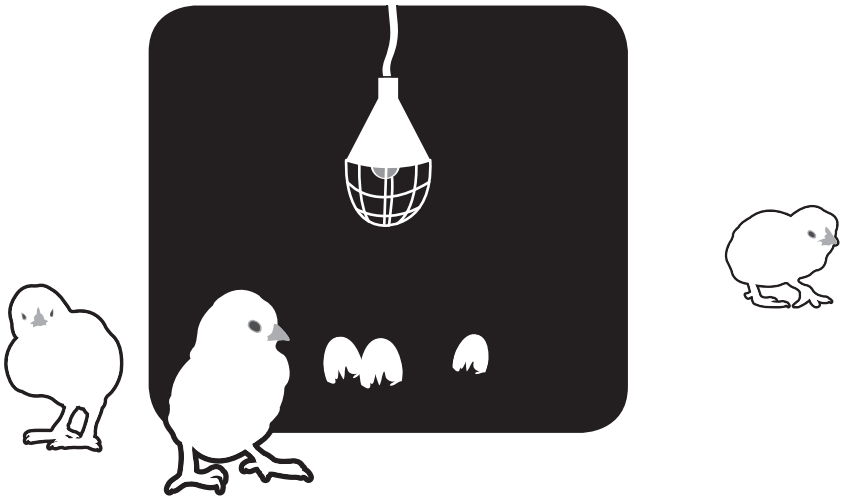
When investigating the adaptive capacity of animals to infectious challenges, the immune system plays an important role and may also represent a common ground between animals of different early life conditions. In this thesis, we measured immune responses in Chapter 2, 5 and 6, and the results demonstrated that both the adaptive and innate immune response were influenced by early life conditions. Effects of early life conditions on the adaptive immune response were found when layers were either immunologically immature (< 8 weeks of age; antibody titre to *E. acervulina*, Chapter 6) or when the antigen was encountered before (IB titre in later life after vaccination in early life; Chapter 2). Effects on innate immune parameters were found from 8 weeks of age onward, as we found differences in macrophage area after *Eimeria* at 8 weeks of age (Chapter 2), and in natural antibody titres to LPS and KLH after 8 weeks of age (Chapter 5). This may be an indication that, dependent on the age of the layers at the time of the infectious challenge, either the innate or adaptive immune response is more important. However, this statement would require further investigation.

CONCLUSIONS AND IMPLICATIONS

The aim of this thesis was to investigate whether contrasts in early life conditions could influence the adaptive capacity to an infectious challenge in the rearing period, and whether this might be an alternative method to improve animal health and disease resistance instead of the current strategies of disease control and prevention (Ten Napel et al., 2006).

It can be concluded that early life conditions can indeed influence the ability of layers to respond to an infectious challenge later in the rearing period. Based on the results presented in this thesis, it seems that optimized early life conditions of layers, i.e. optimal incubation temperature, hatching with early feed availability within 6 h, and enrichment of the rearing environment, have a positive effect on the adaptive capacity during infectious challenges. However, it has to be taken into consideration that the type and of infectious challenge (e.g. parasite, virus, bacteria), its virulence, but also the timing of the challenge (e.g. during immune maturation process or not) and the life history of the animal are all important factors that determine the effectiveness of the adaptive response to the challenge. The results in this thesis demonstrated that layers with a different life history may have different strategies to adapt to the infectious challenge, based on what is most favourable for them considering their body condition, stress responsiveness etc., but also considering their current environment.

It also has to be taken into consideration that the infectious challenges applied in this thesis were relatively mild (i.e. no mortality was observed), and it remains difficult to assess how the adaptive capacity would be affected during more severe infectious challenges. Moreover, in the experiments performed in this thesis, layers of the same line (Lohmann Brown classic) were housed indoors in controlled conditions, whereas conditions in commercial circumstances may not be as controlled and housing systems may even have an outdoor range. It is therefore recommendable to conduct further research on a commercial scale to gain more insight in the potential of early life conditions to influence the adaptive capacity during infectious challenges in varying layer lines and circumstances. However, implementation of the early life conditions as described in this thesis in commercial husbandry systems will be the first step towards an alternative method to improve animal health and disease resistance in the animal production sector instead of the currently used prevention and control methods.



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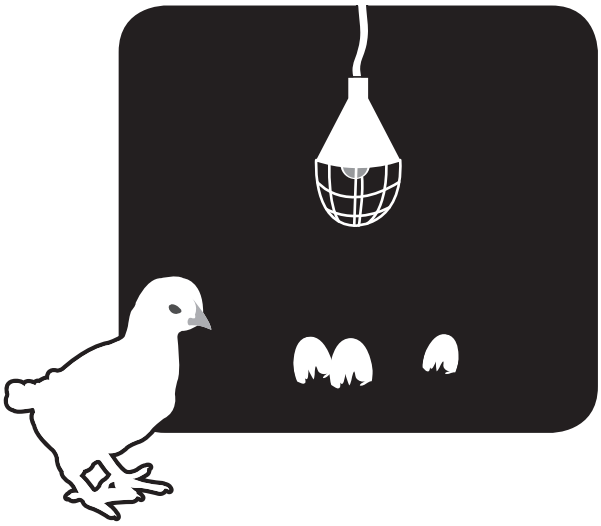


ENGLISH SUMMARY

The current strategy to deal with pathogens in the layer industry is based on prevention and control methods and primarily aimed at minimizing the risk of infection with the pathogen. The aim of this thesis was to investigate a possible alternative strategy for maintaining layer health and controlling diseases, by utilizing the intrinsic adaptive capacity of layers for maintaining their own health and welfare during (infectious) challenges. The adaptive capacity already develops early in life and is known to be influenced by early life conditions and experiences. The focus of this thesis was mainly on the adaptive capacity during infectious challenges, in which a good adaptability means that the layer has minimal clinical symptoms and a fast recovery after the challenge.

The first experiment in this thesis (Chapter 2) investigated whether large contrasts in incubation temperature, hatching and rearing environment, as they may occur in practice, could influence the adaptive capacity of layers during infectious challenges, by using the *Eimeria* parasite and the IB virus as model infections. Layers were subjected to suboptimal eggshell temperature (EST 36.7-37.8-38.9°C in week 1-2-3 of incubation) and hatching (hatchlings remained in the incubator until pull out), or optimized EST (37.8°C) and hatching (dry hatchlings were placed in pens with feed, water, foraging material), combined with a cage or enriched rearing environment in the first 7 weeks of life.

Optimized incubation plus hatching followed by enriched rearing resulted in the least weight loss and the highest feed intake after the *Eimeria* infection compared to all other treatments, and the highest BW gain after IB infection compared to chicks housed in a cage environment in the first seven weeks of their lives. Cage reared layers, however, had a higher IB antibody titre after IB infection than enriched reared layers. These results confirmed that the adaptive capacity to infectious challenges could be influenced with management during a short period in pre- or early postnatal life, but that effects last for a considerable time after cessation of the specific management.

Incubation temperature

The experiments described in Chapter 3 and 4 aimed to investigate the influence of incubation temperature on the adaptive capacity during an infectious challenge. A pilot experiment on thermal manipulation was performed to investigate the possibilities to improve the later life adaptability of layers to high environmental temperatures, as a possible tool to improve their general adaptive capacity, but as no long lasting effects were found, thermal manipulation was not used in the remaining experiments of the thesis.

In Chapter 4, layer embryos were subjected to a suboptimal EST (EST 36.7-37.8-38.9°C in week 1-2-3 of incubation), or optimal EST (EST 37.8°C continuously) and half the layers of each incubation treatment were exposed to heat (35°C) from d 33-36 of age. At d 36 of age, all layers were inoculated with *Eimeria acervulina*. Suboptimal incubation reduced heart weight, chick length and navel condition, which are all indicators for chick quality. Suboptimal incubation temperature also appeared to reduce the adaptive capacity to *Eimeria acervulina*. This was demonstrated by tendencies to lower feed intake and BW gain, more duodenal lesions and higher oocyst production after inoculation of *E. acervulina*. Higher lesion scores and faecal oocyst numbers were especially found when suboptimal incubation was combined with heat exposure preceding the infection. These results

indicate that suboptimal incubated layer chickens tend to be less able to cope with an infectious challenge post hatch, especially when exposed to heat prior to infection.

Early feeding and bacterial colonization

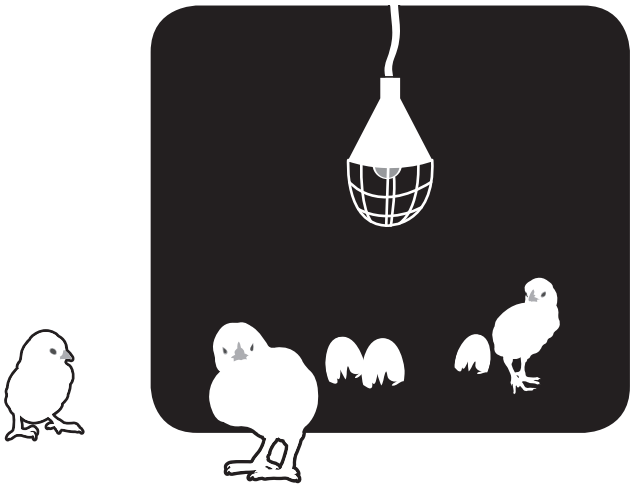
Newly hatched layers in practice have no access to feed and water until arrival at the rearing farm, which may take up to 72 h. The experiments described in Chapters 5 and 6 investigated the effects of feed availability immediately post hatch (early feeding) and manipulation of intestinal microbiota colonization (using early life treatment with the antibiotic Colistin), on growth, microbiota composition and adaptive responses to infectious challenges. Colistin was used in these experiments as a model substance to change (gram negative) bacterial colonization. In both Chapter 5 and 6, newly hatched layers either received feed directly after hatch (F+) or were deprived of feed for 72 h (F-), and were treated with Colistin in their drinking water for 21 d (C+), or received normal drinking water (C-).

In the experiment described in Chapter 5, ileal and caecal samples were taken at 72 h, 21 d and 62 d of age to determine microbiota differences by Denaturing Gradient Gel Electrophoresis (DGGE). At d 62 and d 63 of age, all layers were intratracheally challenged with a 0.5 ml PBS solution containing 0.5 mg of lipopolysaccharide (LPS) + 0.1 mg of Humane Serum Albumin (HuSA) in the trachea and specific and natural antibody levels were determined. Early feeding increased feed intake and BW, changed microbiota composition of the ileum (d 3) and caecum(d 3, 62), reduced the BW gain after challenge and increased T-cell independent (LPS) and natural antibodies (Keyhole Limpet Hemocyanin), but had no effect on T-cell dependent antibody levels (HuSA) as compared to non-fed layers. Colistin treatment resulted in a changed microbiota composition in the caecum at d 3 and 21 and a higher specific LPS titre compared to non-treated layers. From the results we can conclude that early feeding or Colistin treatment: 1) have longer lasting effects on intestinal microbiota composition (both), 2) lead to lasting differences in BW (early feeding), 3) increases specific LPS antibody response (both) and natural antibody response to KLH (early feeding) after a LPS/HuSA challenge, 4) have no effect on the adaptive humoral response determined by the HuSA titre.

In the experiment described in Chapter 6, all layers were inoculated with a 1 mL PBS solution containing 25.000 *Eimeria acervulina* oocysts at d 27 of age, to investigate effects of early feeding and Colistin treatment on the adaptive capacity during an infectious challenge. Early feeding, but not Colistin treatment, resulted in higher BW, heart, liver and bursa weight at 72 h. Moreover, early feeding resulted in a lower BW gain, higher oocyst production per gram faeces and a higher antibody titre, but lower lesion scores in the intestines after *E. acervulina*, and early fed chickens remained heavier throughout infection. Colistin treatment reduced antibody titre and the BW gain of non-fed chickens. From the results it can be concluded that early feeding is positive for growth, but also that both early feeding and Colistin treatment result in changed adaptive responses to an *Eimeria acervulina* infection, indicating that the microbiota composition in early life may play a role in the development of the adaptive capacity of layers.

Conclusion

It can be concluded that early life conditions can indeed influence the ability of layers to respond to an infectious challenge later in the rearing period. Based on the results presented in this thesis, it seems that optimized early life conditions of layers, i.e. optimal incubation temperature, hatching with early feed availability within 6 h, and enrichment of the rearing environment, have a positive effect on the adaptive capacity during infectious challenges. However, it has to be taken into consideration that the type and of infectious challenge (e.g. parasite, virus, bacteria), its virulence, but also the timing of the challenge (e.g. during immune maturation process or not) and the life history of the animal, as well as its current environment are all important factors that determine the effectiveness of the adaptive response to the challenge. It is recommendable to conduct further research on a commercial scale to gain more insight in the potential of early life conditions to influence the adaptive capacity during infectious challenges. However, implementation of the early life conditions as described in this thesis in commercial husbandry systems will be the first step towards an alternative method to improve animal health and disease resistance in the animal production sector instead of the currently used prevention and control methods.



NEDERLANDSE SAMENVATTING

Het huidige beleid voor het bestrijden van dierziekten in de leghennenhouderij is vooral gebaseerd op preventie en controlemaatregelen, met als doel het risico op infectie met een pathogeen te minimaliseren. Het doel van dit proefschrift was om een alternatieve strategie voor het bestrijden van dierziekten te onderzoeken, namelijk door gebruik te maken van het adaptatievermogen van de leghen zelf. De hypothese was dat een goed ontwikkeld adaptatievermogen een leghen in staat stelt om op een adequate manier te reageren op een pathogeen. Het adaptatievermogen ontwikkelt zich al vroeg in het leven en kan worden beïnvloed door vroege omgevingscondities en ervaringen die een leghen in het vroege leven opdoet. Dit proefschrift was vooral gericht op het onderzoeken van het adaptatievermogen tijdens infectieuze stressoren, waarbij minimale klinische symptomen en een snel herstel na de infectie werden gezien als kenmerken voor een goed ontwikkeld adaptatievermogen.
In het eerste experiment is onderzocht of grote contrasten in incubatietemperatuur, uitkomst- en opfokomgeving, zoals deze in de praktijk kunnen voorkomen, het adaptatievermogen van leghennen tijdens infectieuze stressoren konden beïnvloeden. Zowel een mix van verschillende soorten *Eimeria* parasieten als een IB virus werden hierbij gebruikt als model voor infectieuze stressoren (Hoofdstuk 2). Leghennen werden uitgebroed bij een suboptimale eischaaltemperatuur (EST 36.7-37.8-38.9°C in week 1-2-3 van het broedproces) en bij uitkomst in de incubator gelaten tot en met dag 21 van het broedproces, of gebroed bij optimale eischaaltemperatuur (37.8°C) en 6 uur na uitkomst geplaatst in hokken waarin voer, water en strooisel beschikbaar waren. Vervolgens werden de kuikens van beide behandelingen toegedeeld aan twee opfokomgevingen: een opfok in kooien of in verrijkte grondhokken tijdens de eerste 7 weken van hun leven.
De resultaten van dit experiment lieten zien dat optimale incubatie en uitkomst gevolgd door een huisvesting in een verrijkte omgeving resulteerde in minder gewichtsverlies en een hogere voeropname tijdens de *Eimeria* infectie in vergelijking met de andere behandelingen, en daarnaast ook in een hogere gewichtstoename tijdens de IB infectie in vergelijking met leghennen in kooihuisvesting. Leghennen in kooihuisvesting hadden een hogere IB antilichaamtiter tijdens IB infectie vergeleken met leghennen in verrijkte huisvesting.
Deze resultaten bevestigden de hypothese dat het adaptatievermogen tijdens infectieuze stressoren beïnvloed kan worden met management gedurende een korte periode in het pre- en postnatale leven van een leghen, maar dat de effecten nog geruime tijd na beëindiging van dat specifieke management te vinden zijn.

Incubatie temperatuur

De experimenten beschreven in Hoofdstuk 3 en 4 hadden als doel om te effecten van incubatietemperatuur op het adaptatievermogen van leghennen tijdens infecties te onderzoeken. Hoofdstuk 3 werd het effect van prenatale temperatuurmanipulatie op het adaptatievermogen tijdens hoge temperaturen na uitkomst onderzocht, met als doel om uiteindelijk het algemene adaptatievermogen van de leghen te verbeteren. Tijdens dit experiment werden er geen effecten van prenatale temperatuurmanipulatie gevonden, en daarom werd deze methode verder niet meer gebruikt in dit onderzoek.
In Hoofdstuk 4 werden leghenembryo's gebroed bij een suboptimale eischaaltemperatuur (EST 36.7-37.8-38.9°C in week 1-2-3 van het broedproces), of bij een

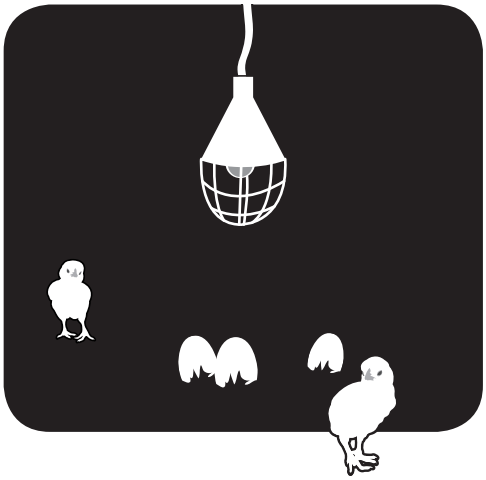
optimale eischaaltemperatuur (37.8°C) en de helft van de leghennen van elke incubatiebehandeling werd blootgesteld aan een hoge omgevingstemperatuur (35°C) van dag 33-36 na uitkomst.
Op een leeftijd van 36 dagen werden alle leghennen vervolgens geïnoculeerd met *Eimeria acervulina*. Een suboptimale eischaaltemperatuur tijdens het broeden resulteerde in een verlaagd hartgewicht, een kortere kuikenlengte en een slechtere navelconditie, wat allemaal symptomen zijn van een verlaagde kuikenkwaliteit. Een suboptimale eischaaltemperatuur tijdens het broedproces leek ook te resulteren in een slechter adaptatievermogen tijdens *Eimeria acervulina*. Dit was af te leiden uit verschillende statistische trends richting een lagere voeropname en lagere gewichtstoename, meer laesies in het duodenum en een hogere oocyst productie na inoculatie met *E. acervulina*. Meer laesies in het duodenum en een hogere oocyst productie werd voornamelijk gevonden in suboptimaal gebroede hennen die waren blootgesteld aan de hogere temperatuur vlak voor *E. acervulina* inoculatie. Deze resultaten zijn een sterke aanwijzing dat een suboptimaal broedproces esulteerde in leghennen die minder goed instaat leken te zijn om te gaan met een infectieuze stressor, vooral wanneer ze voorafgaand aan de infectie werden blootgesteld aan een hoge omgevingstemperatuur.

Vroege voeding en bacteriële kolonisatie
Kuikens in commerciële broederijen hebben na uitkomst geen toegang tot voer en water totdat ze gearriveerd zijn in hun opfokomgeving (24-72 uur later). Hoofdstuk 5 en 6 beschrijven of het verstrekken van voer onmiddellijk na uitkomst (vroege voeding), en het manipuleren van de bacteriële samenstelling in de darm (d.m.v. vroege behandeling met het antibioticum Colistine) invloed hebben op de groei van leghennen, de microbiële samenstelling van de darm en de adaptieve respons tegen infectieuze stressoren. Colistine werd als modelantibioticum gebruikt om de gram negatieve bacteriële kolonisatie van de darmen te beïnvloeden. In zowel Hoofdstuk 5 als Hoofdstuk 6 kregen leghennen direct na uitkomst voer (F+) of pas 72 uur later (F-), en werden ze behandeld met Colistine in hun drinkwater voor 21 d (C+) of kregen ze geen Colistine (C-).
In het experiment beschreven in Hoofdstuk 5 werden monsters genomen van het ileum en caecum van leghennen op 72 uur, 21 dagen en 62 dagen na uitkomst om verschillen in de microbiële samenstelling te kunnen bepalen met 'Denaturing Gradient Gel Electrophoresis (DGGE)'. Op een leeftijd van 62 en 63 dagen werden alle leghennen intra-tracheaal geïnoculeerd met een 0.5 ml PBS oplossing met 0.5 mg lipopolysaccharide (LPS) + 0.1 mg Humaan Serum Albumine (HuSA). Specifieke en natuurlijke antilichaamproductie werd bepaald na de inoculatie met LPS/HuSA. Vroege voeding verhoogde de voeropname en het gewicht van leghennen, veranderde de microbiële samenstelling van het ileum (d 3) en caecum (d 3, 62), verlaagde de gewichtstoename na de LPS/HuSA inoculatie en verhoogde de T-cel onafhankelijke (LPS) en natuurlijke antilichaam productie, maar had geen effect op de T-cel afhankelijke antilichaam productie (HuSA) vergeleken met leghennen die geen vroege voeding kregen. Colistine behandeling had geen effect op groei, maar wel op de microbiële samenstelling van de darm. Uit deze resultaten kan geconcludeerd worden dat vroege voeding of Colistine behandeling: 1) langdurige effecten had op de microbiële samenstelling in de darm (beide), 2) leidde tot langdurige verschillen in lichaamsgewicht (vroege voeding), 3) de specifieke

LPS (beide) en natuurlijke (vroege voeding) antilichaamproductie na LPS/HuSA inoculatie verhoogde, 4) geen effect had op de adaptieve humorale immuun respons (HuSA titer) (beide).
In het experiment beschreven in Hoofdstuk 6 werden alle leghennen op een leeftijd van 27 dagen geïnoculeerd met 1 ml PBS oplossing met daarin 25000 *Eimeria acervulina* oocysten, om te onderzoeken of vroege voeding en Colistine behandelingen het adaptatievermogen tijdens een infectieuze stress beïnvloeden. Vroege voeding resulteerde in een hoger lichaamsgewicht, en hart-, lever- en bursagewicht op 72 uur na uitkomst. Vroege voeding resulteerde ook in een lagere gewichtstoename, een hogere oocyst productie per gram faeces en een hogere antilichaamproductie, maar een lagere lesiescore in de darmen na *E. acervulina*. Daarnaast bleven tijdens de infectie vroeg gevoerde dieren zwaarder dan niet-gevoerde hennen. Colistine behandeling verlaagde de antilichaamproductie en de gewichtstoename alleen in niet gevoerde leghennen tijdens de *E. acervulina* infectie. De conclusie van dit experiment was dat vroege voeding een positieve invloed had op de groei van leghennen, maar ook dat zowel vroege voeding als Colistine behandeling resulteerden in een veranderde adaptieve respons op een *Eimeria acervulina* infectie. Dit is een indicatie dat de microbiële samenstelling van de darm vroeg in het leven een effect kan hebben op de ontwikkeling van het adaptatievermogen van leghennen later in het leven, mogelijk door een beïnvloeding van de (humorale) immuunrespons.

Conclusie

Uit de resultaten in dit proefschrift kan worden geconcludeerd dat condities vroeg in het leven van invloed kunnen zijn op het vermogen van leghennen om te reageren op een infectieuze stress later in het leven. Hierbij lijkt het erop dat geoptimaliseerde vroege condities, zoals optimale eischaaltemperatuur tijdens het broedproces, uitkomst met vroege voeding en verrijking van de opfokomgeving, een positieve invloed hebben op het adaptatievermogen tijdens infecties in het later leven. Er moet echter wel rekening gehouden worden met het feit dat het type pathogeen (parasiet, virus, bacterie), de virulentie, de timing van de infectie (gedurende immuun ontwikkeling of niet), de ervaringen van een leghen voor het moment van infectie en de huidige omgeving waarin een leghen leeft, allemaal van invloed kunnen zijn op de effectiviteit van de adaptieve respons op een infectie. Het is daarom aan te bevelen om ook onderzoek te doen op een commerciële schaal om meer inzicht te krijgen in de mogelijkheden om het adaptatievermogen tijdens infecties te beïnvloeden met vroege condities. Echter, het implementeren van de vroege condities zoals beschreven in het huidige proefschrift is de eerste stap richting een alternatieve methode om diergezondheid en ziekteweerstand te verbeteren in de leghennenhouderij.



CURRICULUM VITAE

CURRICULUM VITAE *(English)*

Irene Walstra was born on March 15 1983 in Leeuwarden, where she was also raised. In 2001, after graduating from Scholengemeenschap Piter Jelles, location Aldlân in Leeuwarden, she started with her study Biology at the Rijksuniversiteit Groningen (RuG).

During her study she had a very broad interest and for the specialisation Behavioural- and Neurosciences, she conducted research at the Animal Physiology and Animal Behaviour Group of the RuG, and went to the Ethology Group of the EÖtvÖs Loránd University in Budapest, Hungary, for a 3 month project in 2006.

Irene Walstra graduated for her MSc in 2007, and in the same year she started as a PhD student at the Adaptation Physiology Group of Wageningen University, in collaboration with the Animal Breeding and Genomics Centre of Wageningen UR Livestock Research. The results of her study are presented in this thesis.

Currently Irene Walstra is working at the University of Applied Sciences Van Hall Larenstein in Leeuwarden.

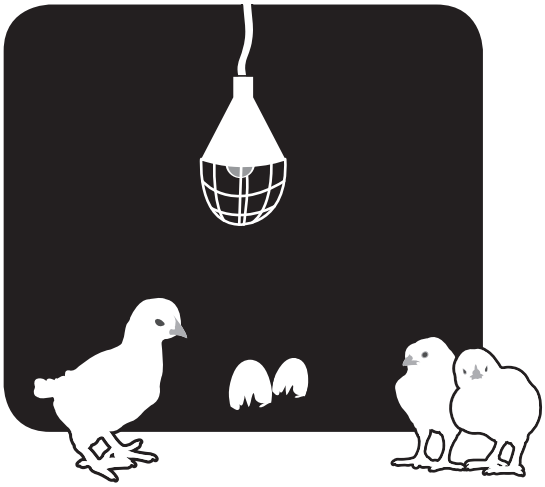
CURRICULUM VITAE *(Nederlands)*

Irene Walstra werd geboren op 15 maart 1983 in Leeuwarden, waar ze ook opgroeide. In 2001, nadat ze haar diploma haalde aan Scholengemeenschap Piter Jelles, locatie Aldlân, begon zij aan haar studie Biologie aan de Rijks Universiteit Groningen.

Tijdens haar studie had zij een brede interesse en voor de specialisatie Gedrag- en Neurowetenschappen heeft zij onderzoek gedaan bij de afdelingen Dierfysiologie en Diergedrag van de RuG. In 2006 is zij voor 3 maanden naar Boedapest, Hongarije geweest om daar onderzoek te doen bij de Ethologie afdeling van de EÖtvÖs Loránd Universiteit.

Irene Walstra behaalde in 2007 haar MSc diploma en in ditzelfde jaar begon ze als promovenda bij de leerstoelgroep Adaptatiefysiologie van de Wageningen University, in samenwerking met het Animal Breeding and Genomics Centre van Wageningen UR Livestock Research. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.

Momenteel is Irene Walstra werkzaam aan de Hogeschool Van Hall Larenstein in Leeuwarden.



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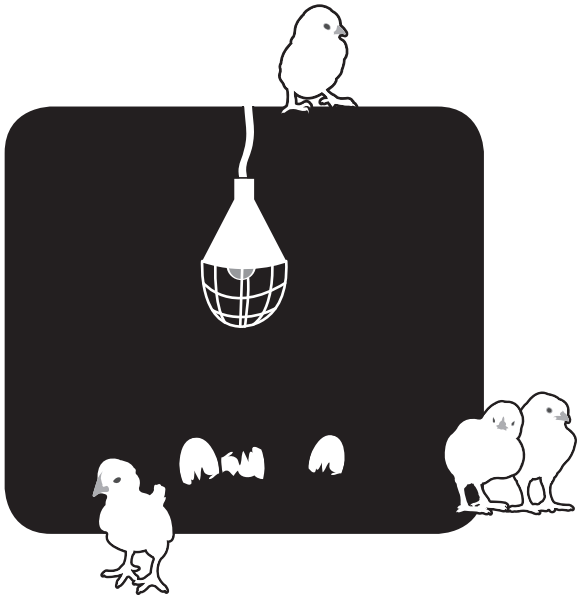


TRAINING AND SUPERVISION PLAN

THE BASIC PACKAGE (3.0 ECTS)

WIAS Introduction Course	2008
WGS course Ethics and philosophy of animal science	2008

INTERNATIONAL CONFERENCES (4.8 ECTS)

9th International Symposium on Avian Endocrinology, Leuven, Belgium	2008
8th European Symposium on Poultry Welfare, Cervia, Italy	2009
4th Workshop on Fundamental Physiology and Perinatal Development in Poultry, Bratislava, Slovakia	2009
Incubation and Fertility Research Group meeting, Tours, France	2010
XIIIth European Poultry Conference, Tours, France	2010
5th Workshop on Fundamental Physiology and Perinatal Development in Poultry, Wageningen, the Netherlands	2011

SEMINARS AND WORKSHOPS (1.8 ECTS)

WIAS seminar Personalities in animals: Implications for welfare	2007
Sectormiddag Legpluimveehouderij WPSA, Wijchen, the Netherlands	2007
Nederlandse Vereniging Gedragsbiologie Meeting, Dalfsen, the Netherlands	2008
WIAS Science Day, Wageningen, the Netherlands	2008 - 2011

PRESENTATIONS (7.0 ECTS)

Poster presentation at Netherlands Society of Behavioural Biology meeting, Dalfsen, the Netherlands	2008
Poster presentation at WIAS Science Day, Wageningen, the Netherlands	2009
Oral presentation at 8th European Symposium on Poultry Welfare, Cervia, Italy	2009
Oral presentation at 4th Workshop on Fundamental Physiology and Perinatal Development in Poultry, Bratislava, Slovakia	2009
Oral presentation at XIIIth European Poultry Conference, Tours, France	2010
Oral presentation at seminar 'The embryonic life of chickens; factors that influence development', Wageningen, the Netherlands	2010
Oral presentation WIAS Science Day, Wageningen, the Netherlands	2011

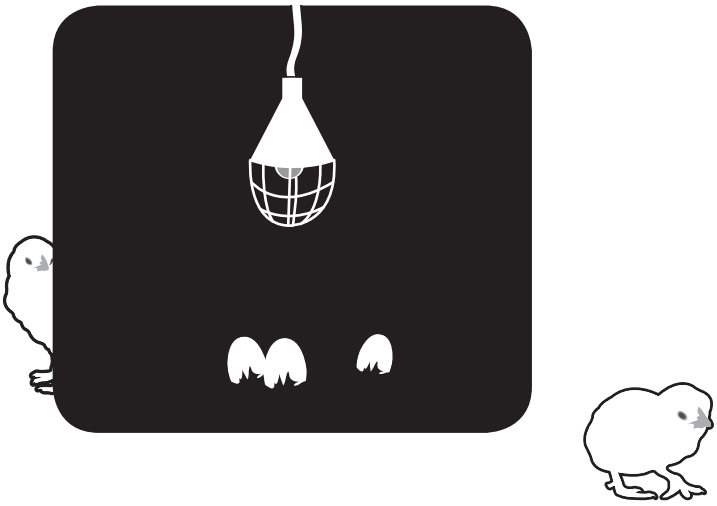
IN-DEPTH COURSES (13.0 ECTS)	
Basic Medical Immunology	2007
ELISA: basic understanding and trouble shooting	2007
Use of laboratory animals (art. 9)	2007
Epigenesis and Epigenetics	2008
Use of laboratory isotopes	2008
Statistics for the Life Sciences	2008
Poultry Discussion Group	2007 - 2008
Interpretation of Animal Stress Responses	2009
Welfare Discussion Group	2009 - 2011

PROFESSIONAL SKILLS SUPPORT COURSES (4.4 ECTS)	
PhD Competence assessment	2008
Teaching and Supervising thesis students	2009
Project and Time Management	2009
PhD Career perspectives	2011

DIDACTIC SKILLS TRAINING (12 ECTS)	
Lecturing in BSc course Adaptatiefysiologie-1	2009 - 2010
Reviewing papers and judging research proposals of Research Master Cluster students	2009 - 2010
Assisting coaching project group Adaptatiefysiologie-2	2009
Assisting project group Inleiding Dierwetenschappen	2010
Supervising 5 BSc-MSc students	2007 - 2011

MANAGEMENT SKILLS TRAINING (8.3 ECTS)	
Member of Platform Gedrag en Welziin	2007 - 2008
Organization of WIAS Science Day	2008
Member of the WAPS Council	2008 - 2010
Member of the Education Committee	2008 - 2009
Organization of 5th Workshop on Fundamental Physiology and Perinatal Development in Poultry	2011

EDUCATION AND TRAINING TOTAL	54.3 ECTS



COLOPHON

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