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A flying start

Adapted hatching and post-hatch feeding in broiler chickens

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Cover: Newly hatched chicks
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Abstract

Conventional hatching practices do not involve provision of feed and water to broiler chickens before placement at the rearing farm. This can pose problems, especially for early hatchlings, which remain feed-restricted for a considerable period due to biological variation in hatch time. The individual requirements of modern fast-growing chickens in order to remain robust and resilient may have changed in recent decades. This thesis studied the effect of hatching time on interval to first feed intake, growth and organ development in chicks hatched on-farm. It also examined the effects of providing hatching chicks with access to feed and water in the hatcher, combined with two different probiotics, on productivity, organ development, immune traits and development of caecal microbiota. Physiological differences between chicks hatched at different times during the hatching window generally levelled out during the production period. Eating-related activities were low immediately post-hatch, and early hatchlings showed compensatory growth as they were lightest at hatch but heaviest at three days of age. Feed deprivation during hatch resulted in depressed feed intake and associated depressed growth, which in some cases was reversed during the growing phase. Supplementation with probiotics had some negative effects on productivity, especially for chicks deprived of feed and water at hatch. There were no long-term differences due to early feeding on microbiota development or immune traits measured. These results suggest that modern broiler chickens are at least partly capable of compensating for setbacks in early life, and that some early effects of feed and water deprivation are transient. However, the studies in this thesis were performed in highly sanitary conditions and at lower stocking densities than in commercial production. Further research is needed to confirm the effects of adapted hatching and post-hatch feeding strategies in conditions that resemble more closely the challenges faced by the modern broiler.

Keywords: adapted hatching, on-farm hatching, post-hatch feed, early feed, eating activity, crop fill, probiotics, immune function, microbiology, gut development

En flygande start. Anpassad kläckning och tidig utfodring till slaktkyckling

Sammanfattning

Konventionell kläckning av slaktkyckling innefattar inte utfodring med foder och vatten förrän kycklingen placerats på gården. Detta kan medföra problem särskilt för tidigt kläckta kycklingar, som på grund av variation i kläcktid blir utan foder och vatten under lång tid. Förutsättningarna för dagens snabbväxande kyckling att förbli robust utan tidig näring kan ha förändrats de senaste decennierna. I denna avhandling studerades hur tiden från kläckning till första foderintag påverkade tillväxt och organutveckling hos kycklingar kläckta i stallet. Därutöver studerades hur kläckning i kläckare med möjlighet att förse kycklingarna med foder, vatten och två olika probiotiska tillskott, påverkade produktivitet, organutveckling, immunparametrar samt utveckling av blindtarmens mikrobiota. Fysiologiska skillnader mellan kycklingar kläckta under olika tidpunkter i kläckfönstret hade jämnat ut sig vid försökets slut. Födosöksrelaterat beteende var lågt i anslutning till kläckning, och tidigt kläckta kycklingar visade prov på kompensatorisk tillväxt då de vägde minst vid kläckning men mest dag tre. Avsaknad av foder och vatten i kläckaren resulterade i lägre tillväxt och foderintag efter utplacering på gården, effekter som i vissa fall lindrades under tillväxtfasen. Tillskott av probiotika hade negativ påverkan på vissa produktivitetsparametrar, effekter som intensifierades när tillskott samtidigt gavs till kycklingar som inte haft tidig tillgång till foder och vatten. På lång sikt sågs inga skillnader härrörande från tidiga utfodringsstrategier på mikrobiota eller immunparametrar. Resultaten tyder på att den moderna slaktkycklingen åtminstone delvis är kapabel att kompensera för tidiga motgångar i livet, samt att tidiga effekter av att inte ha tidig tillgång till foder och vatten i vissa fall är övergående. Studierna genomfördes dock under bättre hygieniska förhållanden och lägre beläggningsgrad än i kommersiell produktion. Vidare forskning krävs därför under förhållanden som mer efterliknar de utmaningar som den moderna slaktkycklingen står inför, för att kunna verifiera effekterna av anpassad kläckning och tidiga utfodringsstrategier.

Nyckelord: anpassad kläckning, gårdskläckning, tidig utfodring, kläckfönster, krävfyllnad, probiotika, immunfunktion, mikrobiologi, vaccin, organutveckling

Dedication

To my beloved parents
Ia & Thomas

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Boyner, M., Ivarsson, E., Andersson Franko, M., Rezaei, M. & Wall, H. (2021). Effect of hatching time on time to first feed intake, organ development, enzymatic activity and growth in broiler chicks hatched on-farm. Animal 15(2), 100083

II. Boyner, M., Ivarsson, E., Wattrang, E., Sun, L., Wistedt, A. & Wall, H. (2023). Effects of access to feed, water, and a competitive exclusion product in the hatcher on some immune traits and gut development in broiler chickens. British Poultry Science, 2163152

III. Boyner, M., Ivarsson, E., Wattrang, E., Sun, L., Wistedt, A. & Wall, H. Improved early growth in broiler chickens given access to feed and water in the hatcher (manuscript submitted to Poultry Science).

Abbreviations

APV	Avian pneumovirus
BA	Biological age
BW	Body weight
CE	Competitive exclusion
ELISA	Enzyme-linked immunosorbent assay
FCR	Feed conversion ratio
FI	Feed intake
GALT	Gut-associated lymphoid tissue
GF	Germ-free
GIT	Gastrointestinal tract
Ig	Immunoglobulin
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry
MALT	Mucosa-associated lymphoid tissue
NGS	Next-generation sequencing
NK cell	Natural killer cell
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid

SLRC	Swedish Livestock Research Centre
TCM	Traditional culture method
YFBM	Yolk-free body mass

1. Introduction

Today's modern broiler chicken and layer hen both originate from the same red jungle fowl (*Gallus gallus*) and later found grey jungle fowl (*Gallus sonneratii*) that are still scratching around the thick tropical forests of India and southeast Asia (Eriksson *et al*., 2008). This wild ancestor is now the closest living approximation to a 'master copy' of the world's most successful domesticated species, which today outnumbers humans 10-fold, with a global population of over 80 billion birds (Gibbons, 2022). According to the Swedish Board of Agriculture, broiler meat consumption has been increasing more than any other type of meat on the Swedish market, with a four-fold increase to 23.1 kg per person and year since the 1980s (SJV, 2021).

Much has happened since the domestication of the red jungle fowl approximately 8000 years ago (Lawal *et al*., 2020). The jungle fowl is a slender neat bird, with males weighing 800-1200 g and females 500-700 g (Endo *et al*., 2021). In its modern counterpart, the growth rate has increased quite exceptionally, *e.g*. in 1957 a broiler chicken weighed 905 g at 56 days of age, while the same rearing time yielded a slaughter live weight of 4202 g in 2005. Since the 1950s, broiler growth rate has increased by over 400%, and feed conversion ratio has improved by 50% in the same period. This profound change in efficiency has been achieved mainly by genetic selection for growth (Zuidhof *et al*., 2014), but also by supplementation of the diet with exogenous enzymes such as phytase (Oakley *et al*., 2014). As another example, within 35 days a modern broiler is capable of reaching a live weight of 2.3 kg by consuming only 3.2 kg of feed (Aviagen, 2022). Thus what the newly hatched chick of today requires in terms of environment and management to maintain robustness, health and productivity may not be equivalent to what it needed some decades ago.

2. Background

2.1 Chicken meat

Worldwide, 101 million metric tonnes of chicken meat were produced in 2022 (Statista, 2023), and broiler meat production is expected to increase globally due to demand for affordable protein sources by the growing global population, which is predicted to reach approximately 10 billion by 2050. In reality, this means that food production will have to increase by 50-90% by that time. Chicken meat production is predicted to increase more than production of other meat types, mainly because of its affordability, acceptability to different religions and suggested health benefits compared with red meat (Zampiga *et al*., 2021).

However, chickens are also responsible for the spread of common human foodborne pathogens (Broom & Kogut, 2018) such as *Salmonella* and *Campylobacter*. It is therefore a high priority for the research community to investigate how chickens in large commercial flocks can remain productive and resilient to pathogens at risk of causing zoonoses. The modern chicken meat industry is quite streamlined, comprising a chain consisting of a hatchery that delivers day-old chicks to a rearing farm, from where the chickens are sent to a slaughterhouse at approximately 35 days of age. Due to this locked chain of actors, there are also great opportunities for small changes in management and environment at any point in the production chain to have a great impact for numerous animals down the line.

2.2 Conventional hatchery practices

In Sweden, the average batch size of broiler chickens for production of meat is approximately 85,000 birds, and the typical chicken producer rears roughly seven batches per year (Swedish Poultry Meat Association, 2022). These chickens start their life at a hatchery, to where eggs from parent flocks are transported and stored before being placed in brooders. The eggs are strictly monitored and kept at a specific humidity, temperature and CO_2 level, and are continuously tipped mechanically, imitating the behaviour of the broody hen. At embryonic day 18, the eggs are ready to be moved to the hatcher, where they hatch within three days (Secher, 2011).

2.2.1 The hatching window

Due to biological differences depending mostly on genetics, but also broiler breeder age, storage time and storage conditions, chicks within the same batch of eggs will not hatch at exactly the same time at the hatchery. A batch of eggs placed in the hatcher at the same time will hatch within a time span of roughly 48 h, which is commonly referred to as the 'hatching window'.

Because conventional hatchers are not equipped to provide feed and water to the chicks, time to first feed intake when the chicks reach the rearing farm can take up to 72 h after quality control, sorting and transport (Willemsen *et al*., 2010). This technical circumstance, combined with set management routines at the hatchery, can pose problems, especially in early hatchlings which will have awaited their later-hatched siblings for quite some time in an environment where feed and water are not available and the temperature is high. For example, one study investigating the effects of feed restriction found that chicks which had to await feed for 48 h lost 6-9 g of body weight, whereas early fed chicks left the hatchery weighing 2.5-3 g more than feed-deprived chicks (Sklan *et al*., 2000). Early hatchlings were the most affected by feed and water deprivation in that study, while late hatchlings were the least affected (Sklan *et al*., 2000). One of the main aspirations for hatcheries is to monitor and decrease the time span of the hatching window, because a broader window adds to the time of feed and water deprivation for new hatchlings, affecting their quality.

2.3 Early chick development

In contrast to the broiler breeding hen, which due to genetic selection and artificial light programmes lays eggs more or less constantly during its 40-week production cycle, the red jungle fowl has a clutch size of 4-6 eggs (Rao *et al*., 2023). The brooding time is the same, 19-21 days, and breeding commences under the influence of increasing hours of daylight during spring and summer. Because of the hatching window, the broody hen is simultaneously responsible for her early hatched chicks while awaiting the arrival of the late chicks, still in their eggshells. Therefore the hen and her clutch have to stay in the nest until the clutch is completed (Moran, 2019). During this time, the mother's life is made easier by the chicks being hatched with a yolk sac embedded in their bellies. This 'packed lunch' is present inside the egg during embryonic life and is made up of 33% lipids, 15% protein, 50% water and less than 1% carbohydrates (Şahan *et al*., 2014). It also contains maternally derived antibodies. The contents of the yolk sac give the chicks a head start before the whole clutch is able to join their mother for longer sequences of feed-seeking activities. Moreover, the antibody transfer *via* the yolk sac provides an immunological safety net before the chicks' own immune system is fully developed (Davison *et al*., 2008).

Because modern broiler chickens grow so rapidly, one day of feed deprivation represents a large proportion of the chick's life and may generate production losses further along the production cycle (Gonzales *et al*., 2003). The yolk sac is very important during the first crucial days, but it has been suggested that it may not be sufficient to support the modern fast-growing chick with nutrients and antibodies in the way that it did for previous generations (Mitchell, 2009). The delay in access to feed and water has a negative impact on growth, organ development, the immune system and digestion, and the length of restriction is of great importance for the outcome. For example, Juul-Madsen *et al*. (2004) found that 48 h of feed and water deprivation impaired growth, immune function and broiler viability, while a 24 h period of feed and water deprivation generated adequate results in terms of growth and immune function. Moreover, a feed-deprived chick needs to mobilise body reserves in order to maintain its metabolism and develop its thermoregulatory system. Naturally, a feed-deprived chick will be more fragile due to lower metabolism and is in greater need of support in terms of additional heat to remain of the same quality as fed chicks (Willemsen *et al*., 2010).

2.3.1 Gut development

At hatch, the chick is dependent on the lipids derived from the yolk sac to maintain its metabolism. Because the yolk sac will be exhausted within 72 h (Mitchell, 2009), the digestive system has to undergo considerable changes in order to convert from intake of an endogenous diet of lipids to an exogenous diet mainly consisting of carbohydrates (Uni *et al*., 1998; Ravindran, 2003). Already during embryonic life, the chick embryo prepares for this transition through initiation of pancreatic digestive enzyme secretion into the intestine of the neonate. Despite this change, starch digestibility immediately post-hatch is low. Early development of the gastrointestinal tract (GIT) is highly dependent on stimuli in terms of feed intake (Gracia *et al*., 2003) and solid feed stimulates the vast morphological changes occurring during the chick's first week of life (Willemsen *et al*., 2010). For example, there is a dramatic increase in mass due to increases in villi number and length and crypt depth (Ravindran, 2003). In parallel with further development of digestive structures, the gut-associated lymphoid tissue (GALT) is rapidly built up (Brisbin *et al*., 2008). GALT is part of the mucosa-associated lymphoid tissue (MALT) and comprises lymphoid tissues. The main types are the caecal tonsils, Peyer's patches, the bursa of Fabricius, Meckel's diverticulum and lymphoid aggregates, which are found at different locations along the digestive tract and harbour different types of immune cells (Brisbin *et al*., 2008).

Different mechanisms are suggested to have effects on the immune system following early access to feed. When feed is provided, substrates needed for physiological processes are added, which may have effects on endogenous hormone levels and other agents that modulate the immune system (Dibner *et al*., 1998). Early-fed chicks are reported to have longer and heavier intestinal segments than feed-deprived chicks, *e.g*. Maiorka *et al*. (2003) observed compromised weights of duodenum and ileum, as well as impaired lengths of jejunum and ileum, already after 24 h of feed deprivation. Moreover, most of the energy and proteins utilised from the yolk is incorporated into the development of the GIT in feed-deprived and fed chicks (Noy & Sklan, 1999). Interestingly, the GIT develops immediately post-hatch regardless of whether the chick is fed or feed-deprived, but early-fed chicks have been shown to have greater intestinal weight than feed-deprived chicks until at least four days of age (Noy & Sklan, 1999). However, for the development of muscle tissue, exogenous feed is essential

(Bigot *et al*., 2003). Even though differences in GIT development between fed and feed-deprived chicks have recently been suggested to be probably short-term and to differ depending on sampling day (Ivarsson *et al*., 2022), there are implications of a start of life where gut development is depressed and delayed. For example, developing and maintaining an immune system consumes energy, so problems may arise if there is a trade-off between body growth and immune function (van der Most *et al*., 2011).

Microbiota and immune function

The gut microbiota has been confirmed in several studies to play an important role in the development and maintenance of the immune system (Ding *et al*., 2017) and for optimal performance and health (Ritzi *et al*., 2016). The microbial community is dynamic and enormous, and spectacularly plastic during the early life of the host. The actual somatic cell count of the host remains roughly the same or is less than the number of colonising microbial cells in most animals, and the number of genes contributed by the microbiota is estimated to be 100 times greater than the estimated amount contributed by the chicken (Ding *et al*., 2017; Broom & Kogut, 2018). There is ongoing and complex cross-talk between host and microbiota that is beneficial for both (Broom & Kogut, 2018; Diaz Carrasco *et al*., 2019). The gut has two main tasks: pathogen defence and nutrient absorption (Oakley *et al*., 2014). The microbiota is beneficial to the host by obstructing pathogens from binding to the gut wall and later colonising the gut, through producing bacteriocins that are toxic to pathogens, and by providing vitamins and energy to the host, through degradation of complex carbohydrates (Broom & Kogut, 2018; Diaz Carrasco *et al*., 2019).

The chick gut was previously considered to be sterile at hatch, but recent evidence suggests that microbial colonisation occurs already during embryonic life, by colonisers at least partly inherited from the maternal hen (Ding *et al*., 2017). The post-hatch chick is very susceptible to diversification of its microbiota, with the microbial abundance rapidly increasing soon after hatch (Ding *et al*., 2017). Once the microbiota obtained has matured, there are only small permanent alterations to it, because an established community will remain resistant to change (Baldwin *et al*., 2018). In a study by Richards *et al*. (2019), the microbiota was found to mature and become more stable between hatch and 21 days of age. The study also found that the microbiota is susceptible to modulation by external factors for more than half of the broiler chicken's life (Richards *et al*., 2019). The time at which the

microbiota is considered stable or matured differs between studies (Bindari & Gerber, 2022), but Li *et al*. (2022b) found that the unstable microbiota in early life is gradually replaced by a more stable microbiota when the chick has adapted to the environment on the rearing farm. During the period of fast skeletal growth (14-21 d) the microbiota remained stable in that study, after which a considerable shift was observed following a change to grower feed and corresponding weight gain (35-42 d) (Li *et al*., 2022b).

Under natural conditions, *e.g.* in red jungle fowl, the chick would have been colonised by microbiota from the surroundings of the mother hen and itself, for instance from nest materials and feathers (de Oliveira *et al*., 2014). Although it is known that the mother hen and the surrounding environment are crucial to establishing a healthy microflora, little is known about the transfer of microbes from the mother hen to its chick. However, Kubasova *et al*. (2019) found that chicks housed together with an adult hen for only 24 h had developed a similar microbiota community to that in the donor hen one week later. Regardless of transfer time, because of the separate upbringing of the breeding parent stock and broiler chickens, natural colonisation is not possible with modern hatching practices. As a result, the chick is prevented from gaining and establishing a commensal microbiota, which in turn might impair its chances of fending off disease (de Oliveira *et al*., 2014; Li *et al*., 2022a).

The intestinal microbiota regulates and modulates the immunological system in different ways, and has effects on both the innate and adaptive immune response, while the immune system in turn has the capability to alter the microbial composition (Kaspers *et al*., 2015). Although the microbiota has effects on both branches of the immune system (innate and adaptive), the microbial community only seems to have a limited impact on the innate immune function, which has been shown to develop naturally even in birds modulated to lack microbiota, *i.e*. germ-free (GF) birds (Kaspers *et al*., 2015). However, there is evidence that the host's initial innate responses can affect specific segments of the microbiota to optimise its composition for inducing subsequent adaptive immune responses (B- or T-cell dependent) (Diaz Carrasco *et al*., 2019). Conversely, absence of microbiota has been shown to have detrimental effects on the development of the adaptive immune system. For example, developmental defects arise in terms of B- and T-lymphocytes being lacking in the gut mucosa in GF birds, leading to failure to produce antibodies (Kaspers *et al*., 2015). Moreover, GF birds are

deficient in an enzyme that is crucial for immunoglobulins to be able to switch classes (*i.e*. IgM to IgA and IgY), which further impairs the flexibility of the immune response and suggests that the complexity of the microbial community is essential to develop a fully matured immune system (Dibner *et al*., 1998; Kaspers *et al*., 2015). The relationship between the host and the microbiota is evidently fine-tuned, and has to be delicately regulated to prevent the host reacting to the microbiota with inflammatory responses (Brisbin *et al*., 2008).

Antibody-mediated immunity

Chickens possess three different types, so-called classes or isotypes, of antibodies. These are: immunoglobulin A (IgA), which is the most abundant in bodily secretions; IgM, which is the first isotype that is expressed during embryogenesis; and IgY, which is the avian homologue and precursor to IgG, the main antibody in mammals (Kaiser & Balic, 2015). Around 75% of the avian immunoglobulin pool is made up of IgY (Chalghoumi *et al*., 2009). In mammals, maternal antibodies that are transferred to the offspring from the mother's bloodstream, either mainly *via* the placenta during foetal life, *e.g*. in humans, or mainly *via* the first milk (colostrum), *e.g*. in ruminants, depending on the type of placentation in each species, are of crucial importance for immune protection of the offspring. The chick is likewise highly dependent on maternally derived antibodies that are transferred from the blood stream of the mother hen and deposited in the egg (Davison *et al*., 2008). When newly hatched and for the first couple of weeks of life, the chick, like mammalian newborns, lacks a fully developed adaptive immune system and is thus dependent on maternally derived antibodies (mainly IgY) to withstand disease-causing pathogens in the immediate surroundings (Davison *et al*., 2008). The mother hen produces antibodies to the antigens she encounters in her environment, which are transferred from her bloodstream and embedded in the yolk of the forming egg. This is quite a sacrifice by the mother hen, which gives up 10-20% of the IgY circulating daily in her bloodstream for the benefit of her chick (Ulmer-Franco, 2012). Because the chick and mother are brought up separately in commercial systems, never crossing paths, the maternal antibodies transferred may not be tailored to the chick's challenges because these may not be the same as those faced by the mother hen. Another consequence of separate brooding and hatching is that the chick will not obtain its mother's microbiota, which would have been matched to the natural antibodies embedded in the yolk.

This discrepancy may leave hatchlings more susceptible to colonisation by undesirable and even pathogenic bacteria (Koenen *et al*., 2004).

Close to hatch, the yolk sac is drawn in and embedded in the belly of the chick embryo (Panda *et al*., 2015). Early access to feed increases the secretion of yolk sac contents into the newly hatched chick's intestine (Noy & Sklan, 2001), further highlighting the importance of early access to feed and water for hatchlings. Although the yolk sac is an important source of nutrients for feed-deprived chicks, it has been discussed that this is not optimal usage of residual yolk, especially since some data suggest that the development of the immune system is highly dependent on availability of exogenous feed at an early stage (Dibner *et al*., 1998). Post-hatch, IgY from the residual yolk sac is transported by the bloodstream, and must reach certain sites to be of full use to the chick. In the chick, IgY is needed to patrol mucosal surfaces, where the risk of invading pathogenic agents is high (Dibner *et al*., 1998). Moreover, as discussed in the previous section on the microbiota, it appears that availability of antigens in the GIT is required to induce full development of the immune system and subsequent chain reactions in terms of developing immune cells such as B-lymphocytes that allow immunological memory to develop (Dibner *et al*., 1998).

There is increasing concern that the intense selection for high body weight in broiler chickens has had unintentional negative effects on the immunological resistance to disease in terms of antibody production (Koenen *et al*., 2004) and reduced adaptive immune functions in meat-type birds (Zerjal *et al*., 2021). Several studies have observed impaired humoral function in fast-growing birds, with the effects of selective breeding seeming to have affected meat-type birds more than layer types (Koenen *et al*., 2002; van der Most *et al*., 2014). The effect of intense selection for high body weight in impairing immune function has been explained by the 'theory of resource allocation', which states that competition for resources by an organism will result in a trade-off when one trait is genetically prioritised over another (Rauw, 2012; Zerjal *et al*., 2021). Decades ago, Qureshi & Havenstein (1994) observed negative effects on the adaptive immune response, with only minor effects on macrophage and NK functions (non-adaptive arm of the immune system) when comparing a 1991 commercial broiler with a 1957 random-bred strain of chicken.

One common cause of economic losses in poultry production is infectious agents that act subclinically and cause poor performance on flock level, as

well as more potent outbreaks of clinical disease that cause high mortality (Sharma, 1999). In Sweden, biosecurity in poultry production is very high, so broiler chickens are not routinely vaccinated. Chicks are therefore dependent on maternal antibody transfer from their mothers, which are vaccinated to the most common diseases (NVI, 2023). A good protective effect is dependent on the chick's ability to start rapidly producing its own antibodies to environmental antigens, an ability that has been shown to be enhanced by provision of probiotics (Brisbin *et al*., 2008).

2.4 Probiotics

The Food and Agriculture Organisation of the United Nations (FAO) defines probiotics as 'live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host' (FAO, 2002). Fifty years ago, competitive exclusion (CE) was introduced as a term to explain how the microbiota limits the incidence of pathogenic microbes, by excluding them physically (from a binding site in the gut) or by excluding them indirectly by occupation of a necessary resource or niche (Nurmi & Rantala, 1973). Although the exact mechanisms for CE have not been identified for all situations, CE is so far the most potent way to prevent colonisation by *Salmonella* in live birds (Oakley *et al*., 2014). In addition to the mechanisms and benefits of CE, probiotics stimulate the development of the immune system and improve nutritional uptake from the gut (de Oliveira *et al*., 2014). Probiotics are commonly used in commercial settings to compensate for the lack of exchange of a healthy microbiota between the chick and adult hen (Seifi *et al*., 2017). Probiotic supplementation appears to be more successful if provided in early life when the microbial community is establishing, or after any disruption of an already established community (*i.e*. stress, dietary change, use of antibiotics) (Oakley *et al*., 2014). Probiotics can have many different effects on the immune system, *e.g*. increased production of antibodies to a given antigen, as mentioned previously (Brisbin *et al*., 2008), and have been observed *e.g*. to boost the immunological response to vaccination for Newcastle disease virus (Kőrösi Molnár *et al*., 2011) and *Eimeria* parasites (Ritzi *et al*., 2016). There are also indirect effects of probiotic supplementation whereby added bacteria can stimulate the growth of other beneficial strains of bacteria, which in turn can have balancing

effects on the microbiota by the production of short-chain fatty acids (Meimandipour *et al*., 2009).

2.5 Alternatives to conventional hatching

The negative effects of feed and water deprivation on growth, productivity, gut health and immune function have been thoroughly reviewed in numerous studies. To alleviate the negative effects of feed and water deprivation and other stressors at the hatchery, *e.g*. exposure to dust, high loads of pathogens (Mitchell & Waltman, 2003), handling (Knowles *et al*., 2004), transport (de Jong *et al*., 2020), darkness (Archer & Mench, 2013) and disinfectants (de Gouw *et al*., 2017), private companies and the research community have begun to investigate alternatives and modifications to conventional hatching. For example, the concept of on-farm hatching has been developed, where fertilised eggs are transported to the rearing farm at embryonic day 18, allowing the chicks to hatch directly in the rearing system, with immediate access to feed and water regardless of their siblings' hatching time (de Jong *et al*., 2020). In Sweden, no conventional producer is currently employing hatching on-farm as a standard procedure, although some organic producers hatch on-farm routinely (Göransson *et al*., 2020). In the Netherlands, *e.g.* the occurrence of on-farm hatching is also low, around 4% in 2019 (de Jong *et al*., 2020). The low rate of conversion to on-farm hatching systems is suggested to be due to the high investment cost and knowledge requirement among producers, but also because the benefits of on-farm hatching with regard to bird health, production and welfare remain unsubstantiated (de Jong *et al*., 2020). Moreover, on-farm hatching systems are labour-intense, which imposes extra costs for the producer. Nowadays, there are also alternatives in terms of hatchers where chicks are hatched under illuminated conditions and where feed and water are accessible immediately post-hatch. Although these alternatives do not remove all disadvantages to chicks of being hatched at a hatchery (*i.e*. dust, pathogens, noise, handling, transport), the negative effects of feed and water deprivation are alleviated. Moreover, light during incubation has been shown to reduce chick susceptibility to stress post-hatch (Archer & Mench, 2013).

3. Aim and Objectives

Although the overall health status of Swedish broiler chickens is considered good, with little need for vaccines and antibiotics, there is always (and should always be, when breeding animals) an endeavour to be better and to make relevant improvements. There is also a general concern that the immune responsiveness of broiler chickens may have been affected by the change in resource allocation brought about by their increased growth capacity over the past couple of decades. Moreover, there has been little research on how new hatching concepts function in a Swedish context. Therefore the main aim of this thesis was to determine whether providing newly hatched chicks with early access to feed, water and bacterial additives can produce more robust broiler chickens during Swedish rearing conditions.

Specific objectives of the work in Papers I-III were to:

- Compare interval to first feed intake, growth and organ development in chicks hatched early, in the middle or late in the hatching window, when hatched on-farm

- Determine the effects of providing chicks with access to feed and water already in the hatcher on growth, feed intake, organ development, gut development and immune function

- Assess the effects of providing chicks with access to two different bacterial additives, through two different administration routes, on caecal microbiota and immune response

4. Comments on Methods

The experiments on which this thesis is based were largely conducted at the Swedish Livestock Research Centre (SLRC) located east of Uppsala, Sweden. In Paper I, eggs were brooded at the hatchery SweHatch, Väderstad, and transported to the research centre on embryonic day 18. Chicks included in Papers II and III were hatched at SweHatch, Lund, where the HatchCare hatcher needed for those studies was available for use. All chickens included in the work were Aviagen's Ross-308 hybrids, which is the most common breed used for chicken meat production world-wide. In all experiments, chickens were housed in modules that were raised from the floor, equipped with wood shavings, three nipple drinkers (Paper I-III) or a bell drinker (Paper III) and a feeder (Papers I-III) (see Figure 1 and Figure 2). The ethical permit for the studies allowed a maximum stocking density of 30 kg/m^2, but the stocking density was even lower in practice due to removal of birds for sampling and lower finishing weight than stated in the ethical application. European Union regulations allow a maximum stocking density of 33 kg/m^2, with the possibility for an increase to 39 or even 42 kg/m^2 on fulfilling certain criteria (Council of the European Union, 2007). For detailed descriptions of the materials and methods used in the experiments, see the respective papers. General comments on some of the methods used are provided below.

4.1 Paper I – Time to first feed intake

The objective of the study described in Paper I was to evaluate the effects of hatching time on chicks' time to first feed intake, organ development, digestive enzyme activity and growth. A total of 400 fertilised, candled and confirmed viable eggs were transported to SLRC on embryonic day 17.5 and hatched on-farm (Figure 3). Organs were collected for measurement of

length and weight at five different ages, and samples for analyses of digestive enzymes were taken on the same occasions. Behaviour observations and crop fill assessments were carried out during the first 36 h and 52 h, respectively, post-hatch. The chicks were divided into three hatching treatments (early, mid-term or late) based on when they hatched within the hatching window. Each hatching treatment was replicated five times, giving 15 modules in total. The chicks were kept for a full production cycle of 34 days.

4.2 Papers II and III – Feed, water and probiotics in the hatcher

The objective of the studies described in Papers II and III was to evaluate the effects of hatching chicks in a hatcher providing access to feed, water and, in addition, one of two bacterial additives (the CE-product Broilact® or the synbiotic product PoultryStar®). Two administrative routes were tested for Broilact®, which was provided either in the drinking water (CE_W) or sprayed on the chicks (CE_S), while PoultryStar® was provided only in the water (PS). The above-mentioned treatments were evaluated relative to a negative (no access to feed and water during hatch; CN) and positive control group (access to feed and water, but no feed additive during hatch; CP). The studies involved a total of 450 chicks hatched in the HatchCare® hatcher. Post-hatch, all chicks were transported to SLRC and provided with feed and water immediately after placement. Thereafter, all groups except the Broilact® hatching groups were divided in half and one half was given access to PoultryStar® for three consecutive days according to the manufacturer's recommendation. The recommended administration of PoultryStar® is flexible, but the product is suggested to be administered during stressful periods in production, which in Paper III was the placement of chicks at the SLRC after transport. The Broilact® treatments were evaluated together with the controls in Paper II, while the PoultryStar® treatments were evaluated together with the controls in Paper III. For Paper III, the three hatching treatments and two post-hatch treatments generated a 3x2 factorial design. At 11 days of age, all chicks were given an intramuscular injection to the breast muscle with a commercial vaccine against avian pneumovirus (APV). Each week, blood was collected from three focal birds per module, and later analysed for both total IgY in serum and antibody response to the APV vaccine. Organ samples were collected from a sample of birds per treatment

on three occasions (on arrival, and at 11 and 32 days of age) and used for measurements and collection of tissue for histological analyses. Caecal samples were collected at the same time and analysed for microbiota composition. The chickens were kept for a whole production cycle of 32 days and body weight (BW) and feed intake (FI) were recorded weekly and used to calculate feed conversion ratio (FCR).

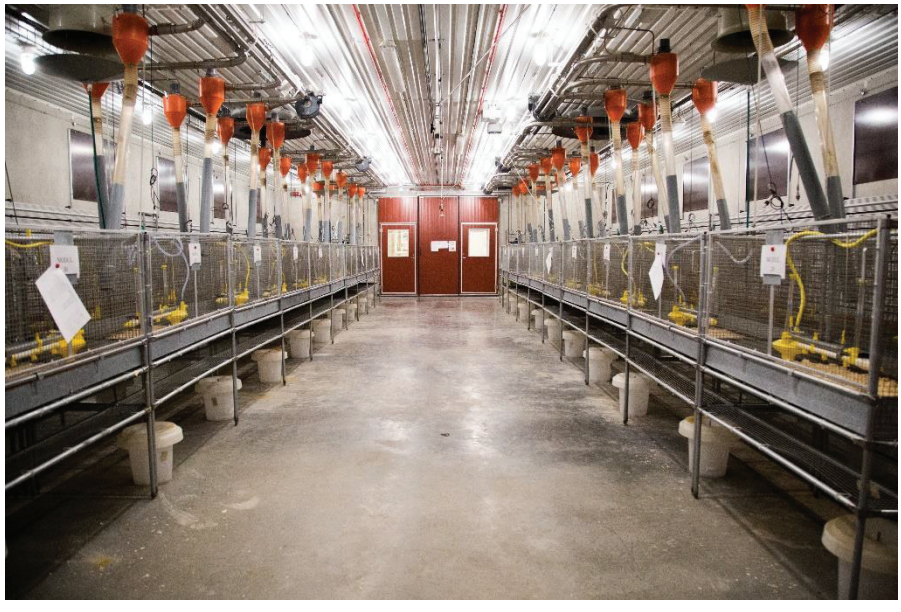

Figure 1. Broiler facility at the Swedish Livestock Research Centre, Uppsala Sweden. Photo: V. Vallejo.

Figure 2. Pen in which the chickens in Papers I-III were kept. Photo: V. Vallejo.

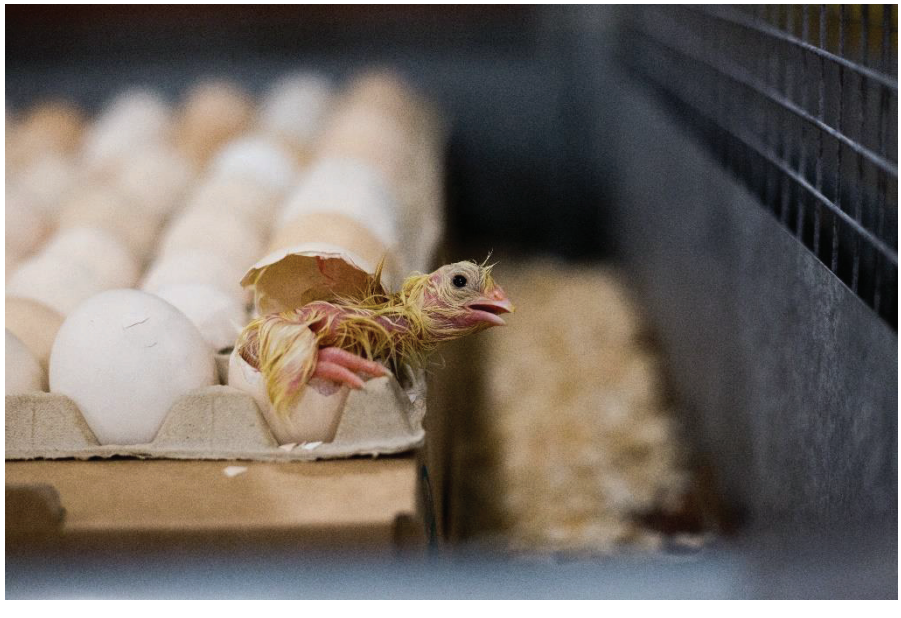

Figure 3. Chick hatching on-farm at 21.53 h on 17 October (Paper I). Eggshell temperature was recorded regularly using an ear thermometer on the egg's equator prior to hatch. Eggs were placed in classical egg trays in each module, which were removed when the chicks had cleared their eggshells. Photo: V. Vallejo.

4.3 Recordings

4.3.1 Behavioural observations and crop fill assessment

In Paper I, observations were performed on hatchling eating behaviour and crop fill in the first couple of days. One common way to observe animal behaviour is to use cameras and analyse the footage digitally. Due to the labour-intensive hatching and placement of chicks, scan sampling was selected as the method of choice in Paper I. When using scan sampling, one or more behaviours of an individual or a group of animals are recorded instantaneously at sequential pre-set points in time (Lehner, 1992). A possible disadvantage with scan sampling is the risk of animals reacting differently to disruptive events, *e.g.* doors opening and a person that could be perceived as frightening approaching and standing close to the module. In Paper I, the personnel performing the observations and assessments had been in the research facility for several days, and also handled the chicks during placement post-hatch. The chicks did not show any signs of being disturbed by the scan sampling. Crop fill assessment was chosen as a follow-up measurement to the behaviour observations, as a further check of whether chicks had started to eat or not. Crop fill assessment is a well-proven method that is routinely performed under commercial conditions to confirm whether chicks have found feed and water (Aviagen, 2018).

4.3.2 Methods to assess immune function

To assess immune function, the chosen method was to quantify the transfer of maternal IgY from mother to offspring and monitor the kinetics of use and/or decay of maternal IgY in the chicks. Moreover, the onset of the chicks' own IgY production and their capacity to respond with antigen-specific antibody production to vaccination were monitored. This was made possible by the frequent blood samplings in Papers II and III and was used as an indicator of overall immunological state of the birds. Early access to feed and water has been suggested to increase the utilisation rate of the yolk sac (Noy & Sklan, 2001). Because the maternally derived antibodies reside there, it was hypothesised that providing feed and water early on would increase uptake of these very important immunoglobulins by the newly hatched chicks. The particular vaccine antigen used (APV) was chosen because the chicks' mothers had not received it, and therefore the chicks did

not have maternally derived antibodies to it. The ability of the chicks in each treatment group to develop a specific response to this antigen was assessed.

Two different types of ELISA methodology (immunoassay) were used to evaluate the IgY level in serum and the antibody response to a known antigen (APV). In immunoassays in general, the capacity of antibodies to bind to a specific structure (epitope) on a target (antigen) is used for different detection purposes. In ELISA, the binding of antibodies to antigen is visualised by an enzyme that generates a colour reaction which can be measured.

The approach used in this thesis to assess chicken immune function focused heavily on antibodies and antibody production, which can be considered a small part of the immune system. However, as mentioned, the transfer of maternal IgY is a very critical part of immune protection in the newly hatched chick. Moreover, antibody production to a novel antigen is the end-product of an immune response that requires the correctly orchestrated functions of a number of immune mechanisms, *e.g.* antigen uptake and presentation by antigen-presenting cells and T-cell activation to initiate T-cell help for B-cells to efficiently produce antigen specific antibodies. Antibody production can thus be considered a more general measure of the function of specific immunity and was chosen in Papers II and III because it evaluates functions of specific immunity that are relevant for the young chick, using easily performed and low-cost methodology (*i.e.* ELISA assays). Alternative immune traits that could have been used include *e.g.* monitoring of leukocyte counts in blood (Wattrang *et al*., 2020, 2022) for a general reflection of immune status, assessment of antigen-specific T-cell responses, as recently described for chickens (Wattrang *et al*., 2022, 2023), and functional tests of innate functions such as phagocytosis by chicken immune cells (Naghizadeh *et al*., 2019). Although these methodologies are available, their use in Papers II and III would have required substantial labour-intensive and costly efforts for set-up of methods for each study and for performing the analysis during the studies. Their use was therefore deemed beyond the scope of the present work.

4.3.3 Sequencing and bioinformatics

After DNA extraction from caecal contents, the 16S rRNA gene was sequenced on the Illumina HiSeq 2500 platform at Novogene (Beijing, China). The 16s rRNA gene is optimal for sequencing, since it codes for a small subunit that can be found in all bacterial ribosomes. In brief (see Papers

II and III for more details), the V3-V4 region of the 16S rRNA gene was amplified with primers together with Phusion® High-Fidelity PCR Master Mix (New England Biolabs), a product that helps with both the speed and fidelity of DNA replication. After quality filtering and trimming, bioinformatics processing was applied to the sequenced data and the amplicon sequence variants (ASV) obtained were assigned taxonomy by comparison to databases. Further analyses of the sequenced data involved determination of relative abundance at genus level, rarefaction curves of observed ASV and principal coordinate analysis of generalised UniFrac distance matrix. As with all methods, there are limitations to these approaches. With 16S rRNA gene amplicon sequencing, most bacteria can only be identified on genus level because the primers bind into regions that are not fully sustained across all bacterial species. The high resemblance in the 16S rRNA gene therefore makes it difficult to distinguish between species that are closely related (Gupta *et al*., 2019). To obtain higher resolution, a metagenomics approach can be used instead, *e.g.* Durazzi *et al*. (2021) found that shotgun metagenomics sequencing detected a greater part of the microbiota community than 16S sequencing and identified less abundant taxa to a higher degree. However, the metagenomics approach is more costly and knowledge-demanding, and requires more advanced bioinformatic resources (Durazzi *et al*., 2021). Before the 1970s, identification of bacteria was reliant on traditional culture methods (TCMs) where bacteria are cultivated on petri dishes in a laboratory, using a nutrient-intense medium (commonly agar or broth) adjusted depending on bacterial type and purpose of the analyses (Gupta *et al*., 2019). Bacterial strains are then analysed under a microscope and identified in further downstream analyses that nowadays may include polymerase chain reaction (PCR) methodology or matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS). Limitations of TCMs are that they are only able to identify a small proportion of the microbiota present in a sample. In addition, some bacteria may flourish under laboratory conditions while others may be unable to grow or may act differently during controlled conditions, which could impose bias.

Next-generation sequencing (NGS) of 16S rRNA can determine the relative abundance of all bacteria in a sample, irrespective of whether a bacterial strain is culturable or not, and is thus a very powerful method that provides reliable and rapidly obtained information (Gupta *et al*., 2019). In

Papers II and III, the objective was to obtain a comprehensive view of the microbiota in chicken caeca, so NGS of the 16S rRNA gene was chosen as the method. If the objective had been *e.g*. to isolate a pre-determined strain of bacterium or study the behaviour of a particular bacterial strain, TCM (or qPCR) might have been preferable.

4.4 Statistics

Productivity measures (BW, FI, FCR), organ weights and lengths, digestive enzyme data and histology data were all evaluated using the Procedure Mixed (PROC MIXED) statement in the statistical software programme SAS (version 9.4), with hatching treatment and age as fixed factors and module as random factor. In cases where chicks were not yet assigned to modules early on in studies, module was not included in the random statement. Organ weights during grow-out were analysed with age as an additional fixed factor and a repeated statement. The unstructured UN covariance structure was used in the first instance, and replaced with the first-order autoregressive AR(1) when necessary. For eating behaviour and crop fill, the statistical software R was used, and 95% confidence interval and odds ratios were estimated, respectively, with respect to biological age (BA). Biological age was defined as 'time elapsed in hours since hatch of the median chick in that group'. For eating activity, a mixed logistic regression model with module as a random factor and a smooth spline component with respect to time from observation start was used. Crop fill data were analysed using a mixed ordinal regression model assuming proportional odds, with module as a random factor and observation time as a categorical variable. Antibody data were presented as mean values with 95% confidence intervals, and if the intervals were non-overlapping were considered as rejecting the null hypothesis of no difference. To check for differences in proportions of positive and negative responders to APV between treatment groups, Fisher's exact test was performed. Microbiota data were analysed at phylum, class, order, family, genus and ASV level, using ANCOM methodology (Mandal *et al*., 2015) to evaluate differences in age and treatment between treatment groups. In Paper II, a difference in microbiota between the treatment groups was observed at day 11 and this was further investigated by selection of genera with relative abundance higher than 1% using the rarefied ASV table.

The selected genera were further analysed in R, using quasi-Poisson generalised linear models.

5. Main Results

The main results obtained from the experiments in Papers I-III are described in brief below. For a complete description of the results, see the respective papers.

5.1 Paper I

5.1.1 Hatching time and eating activity

Chicks in Paper I were divided into three groups, early (476-496 h post incubation start), mid-term (496-504 h) and late (505-511 h), within a hatching window of 35 h. Active eating behaviour was determined as when a chick was either eating or standing close to the feeder. A 5% proportion of chicks performing active eating behaviour was reached at different biological ages (BA) in the different hatch groups. It was reached earliest in late hatchlings (BA 21.7 h), followed by mid-term hatchlings (BA 25.1 h) and was reached latest in early hatchlings (BA 29.5 h). The crop fill assessment data followed the same pattern, where all focal chicks in the mid-term (BA 32.4 h) and late hatch groups (BA 30.5 h) had either half-full or full crops earlier than to the early hatchling focal chicks (BA 40.6 h). The time required for 50% of focal chicks to fill their crop was approximately the same for all hatch groups.

5.1.2 Organ development

At hatch, there were some physiological differences between chicks from the different hatch groups. A lighter small intestine was found in early and late hatchlings compared with mid-term hatchlings. Late hatchlings also had shorter intestines (in relation to body weight) than early and mid-term

hatchlings. Mid-term hatchlings had heavier bursa than early hatchlings. The only remaining difference throughout the grow-out period was that early hatchlings had relatively lighter intestines compared with late hatchlings and a tendency for lower relative intestinal weight compared with mid-term hatchlings.

5.1.3 Production performance

There were no differences in FCR between the hatching groups at hatch or later during the study. From hatch until 3 days of age, there was an effect of hatching group on BW, where late hatchlings were heavier than early and mid-term hatchlings. At 3 days of age, however, early hatchlings were heavier than those in both other groups. The differences in BW were no longer apparent from 10 days onward, although early hatchlings were numerically heavier at the end of the study.

5.2 Papers II and III

5.2.1 Production performance

In Papers II and III, feed deprivation during hatch had depressive effects on feed intake and growth to varying extents (Figure 4). In Paper II, chicks in the negative control group had lower BW from 2 to 11 days of age compared with chicks in all other groups. At 25 days of age, the difference in BW persisted only between the negative and positive control groups, and was no longer apparent at the end of the study. The lower BW was accompanied by lower FI in the negative control group compared with all other groups at 11 and 18 days of age. The negative control group had lower FI throughout the study compared with the positive control group and the Broilact® spray group. Birds in the Broilact® in water group had an inferior FCR than those in all other groups at 25 and 32 days of age. In Paper III, the negative control group, which had access to PoultryStar® in the research facility, showed poorer FCR at 25 days of age compared with all other groups. It also showed lower body weight at 32 days of age compared with all other groups except for that which received synbiotics both at hatch and as a post-hatch treatment, and the negative control group, which did not receive any post-hatch treatment. Both negative control groups in Paper III showed lower FI from 11 days until the end of the study compared with all other groups except for

that which received synbiotics both as a hatch and post-hatch treatment (day 25 and 32).

5.2.2 Organ development

There were some differences between hatch treatments regarding organ weights and lengths at placement, although none of these differences persisted throughout the studies in Paper II and III. In Paper II at 2 days of age, there was a tendency for lower yolk-free body mass (YFBM) in the negative control group compared with all other groups, whereas the negative control group had lower BW and YFBM compared with the other groups in Paper III. Moreover, birds in the negative control group had lower proportional weight and length of intestines compared with those in the other hatch treatment groups in both Paper II and Paper III. In Paper II, birds in the negative control group had heavier gizzards (when weighed emptied and rinsed) compared with those in all other groups, whereas in Paper III positive control birds had lighter gizzards than birds in all other groups. Regarding relative gizzard weight with contents remaining, the values tended to be lowest for the negative control group and highest for the positive control group in Paper II, while a similar, but statistically significant, relationship was found in Paper III. Birds receiving the PoultryStar® hatching treatment did not differ from the controls in terms of full gizzard weight. In Paper III, the feed-deprived chicks were taller than other chicks at placement.

5.2.3 Gut development and immune traits

In Paper II, there was an effect of treatment on microbiota composition at genus level on day 11, where seven bacterial genera were found to differ between the treatment groups. There were no longer any treatment-related differences remaining at the end of that study and no microbiota differences related to treatment were observed in Paper III. No effects of hatching treatment on any of the immune traits measured were observed (Papers II and III). Poor immune response was observed in general with regard to total serum levels of IgY (Figure 5) and in terms of number of positive responders to the vaccine (Papers II and III).

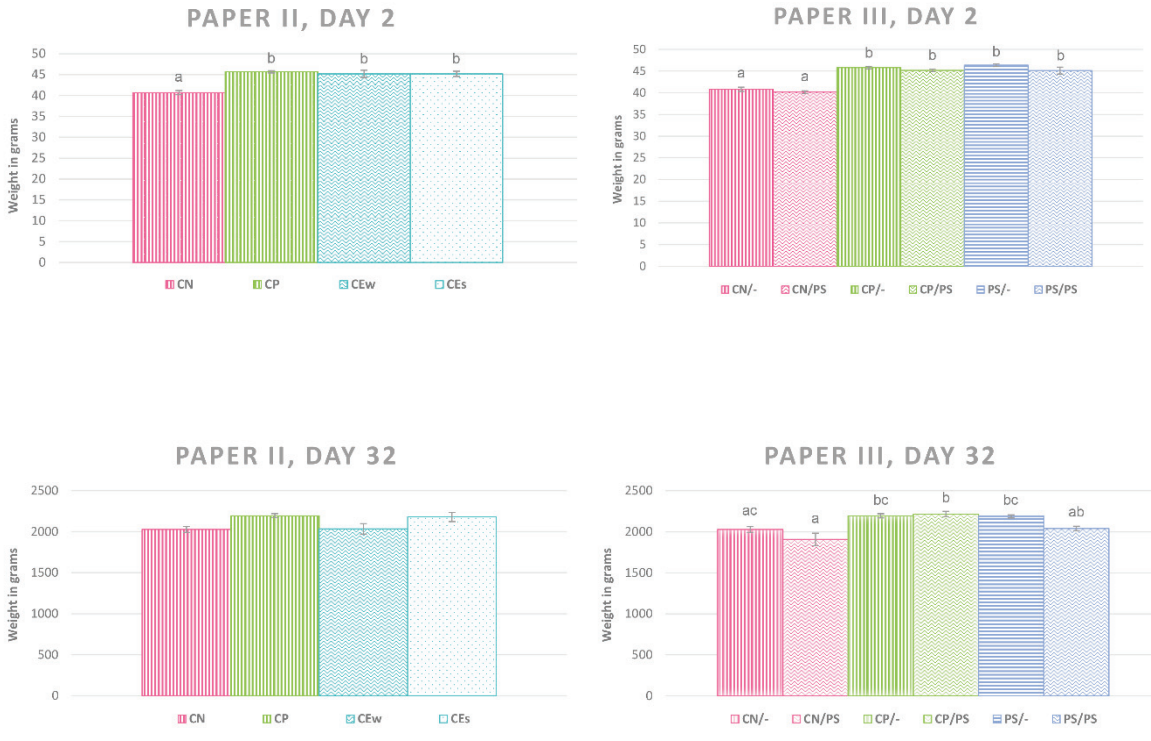

Figure 4. Chicken body weight at 2 and 32 days of age in (left panels) Paper II and (right panels) Paper III. Striped vertical bars represent control hatch treatments (red = no feed or water, green = feed and water). The striped horizontal bar in Paper III diagrams represents hatch treatment (blue = feed, water and synbiotics). Bars with wavy lines represent treatments where bacterial cultures provided in water (CE product in Paper II, synbiotics in Paper III) and the dotted bar a treatment where bacterial culture was provided as an aerosol (CE product, Paper II). Note: chicks weighed at day 2 in Paper III had not yet been given access to their post-hatch treatment. Different superscripts on bars indicate significant differences. Values are least squares means ± SE.

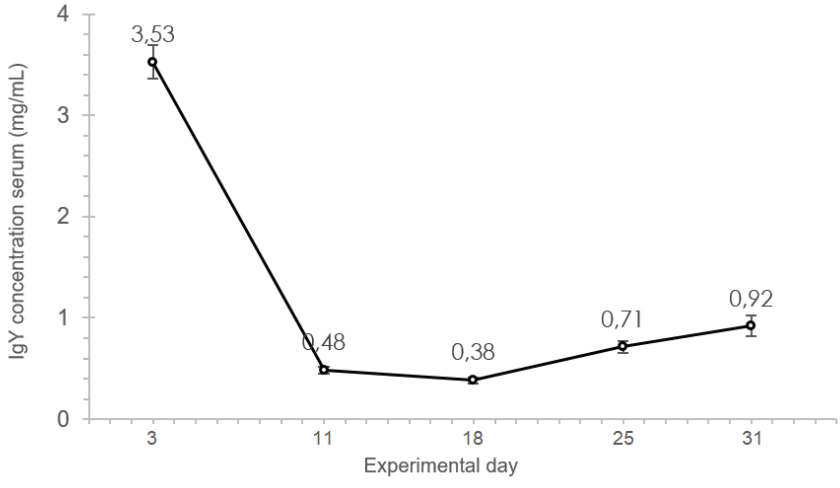

Figure 5. Immunoglobulin-Y (IgY) concentration in serum samples from chickens in the different treatment groups in Paper II and III on five different sampling occasions (at 3, 11, 18, 25 and 31 days of age). Values are mean values for all treatment groups ± 95% confidence interval (horizontal bars).

6. General Discussion

The studies on which this thesis is based (Papers I-III) were carried out to investigate whether adapted management in terms of different hatching concepts, combined with immediate access to post-hatch feed and to bacterial additives in early life, could generate a robust and resilient chicken under Swedish conditions. This chapter discusses the most important results, together with relevant scientific literature. The scientific contributions of the thesis and future implications of work are reported in Chapters 7 and 8, respectively.

6.1 Time to first feed intake

In Paper I, chicks hatched over a hatching window of 35 h, but in conventional hatchery practice the hatching window would have been shorter, for several reasons. According to Aviagen, the company that develops and markets the most common hybrid (Ross 308) sold world-wide, a hatching window of 30 h can be expected for 1 to 99% of the chicks to hatch (Tullett, 2009). In Sweden, a 30 h hatching window is considered normal, but 24 h is achievable with good conditions in storage and handling of eggs, brooding and hatching (M. Backenheim, pers. comm., 22 March 2023). Moreover, the hatching process is terminated at a pre-set time in the hatchery in order to meet the nutritional needs of the early hatchlings and because chicks hatched much later than the pre-set time are less likely to be of high quality, an indicator of this being trouble to break through the eggshell.

Chicks hatched at different time points during the hatching window have been observed to differ from each other physiologically (van de Ven *et al*., 2013; Lamot *et al*., 2014) and behaviourally (Nielsen *et al*., 2010), which

was also the case in Paper I. In Paper I, the chicks had immediate access to feed and water once they had exited their eggshell. Many studies have reported adverse effects of prolonged time to first feed intake, *e.g*. 48 h (Juul-Madsen *et al*., 2004) or 72 h (Bar Shira *et al*., 2005). The majority of the chicks hatched annually worldwide are hatched in conventional hatchers, which are not equipped to handle feed and water provision, and therefore the time to first feed may be as much as 72 h post-hatch (Willemsen *et al*., 2010). In Sweden, the time to first feed intake for the average chick (hatched at the peak of the hatching window) is about 25 h (M. Backenheim, pers. comm., 22 March 2023). The problem with prolonged time to first feed intake is generally expected to affect early hatchers the most, since they appear to be most prone to dehydration and inadequate uptake of the yolk sac during the wait (Sklan *et al*., 2000). However, in Paper I, chicks were observed to be quite exhausted after emerging from the egg, and prioritised sleeping until their down had dried before taking any interest in eating-related activities at all. Only 5% were engaged in eating-related activities at a biological age of 25.4 h, while a biological age of 30.6 h was required before 50% of the chicks had a full crop. However, it should be noted that the chicks in Paper I were hatched in a very calm environment at low stocking density. Hatchery-hatched chicks in commercial conditions, and even on-farm hatched chicks if hatched at full production scale, might have been stimulated to start searching for feed earlier. In addition, the scan sampling methodology used in Paper I captured the activity momentarily, and was not an exact measure of behaviour. However, it was complemented by data from the crop fill assessments, the results of which are more reliable.

These findings suggest that on-farm hatching systems may be of greatest value for farms that are located far from the hatchery, where transportation time might lead to an intensification of problems that arise from feed and water deprivation.

Time to first feed and water intake in modern commercial broilers merits further investigation, as knowledge is lacking on this issue. The time to first feed intake in chicks is suggested to be a matter of only a few hours (de Jong *et al*., 2017), but in one study chicks were shown to have very limited feed intake before 3 days of age (Hogan, 1971). However, the latter study was performed on jungle fowl, the ancestral bird that has far lower metabolic rate and genetic incentives for growth than the modern broiler. Nevertheless, pecking behaviour has been shown to start much earlier than the actual

ingestion of feed, and the chick learns how to distinguish between feed particles and miscellaneous materials peck-by-peck. Interestingly, Hogan (1984) found that during feed deprivation, pecking rate was only affected after two behaviours (pecking and ingestion) had been correlated in the bird. In other words, at hatch there is no signalling between hunger and the pecking system and this develops only after appropriate experience. This suggests that chicks should not be given access to feed in the hatcher only to have it withdrawn during transport, as was the case in Papers II and III.

Although on-farm hatching may be of greatest benefit for chicks that are hatched far from the rearing farm, de Jong *et al*. (2017) found beneficial effects on welfare and production performance in on-farm hatched chicks compared with hatchery-hatched chicks even with short hatchery-farm distances in the Netherlands. However, it is possible that differences between on-farm and hatchery hatching other than feed availability *per se* (*e.g*. handling, noise, high pathogen loads and deprivation of light) affect the outcome. For example, early exposure to dust and disinfectants during conventional hatching is reported to aggravate the negative effects of feed and water deprivation in early life (de Gouw *et al*., 2017).

6.1.1 Differences between early, mid-term and late hatchlings

In Paper I, early hatched chicks reached 5% eating activity and had half-full or full crops at an older biological age than chicks in the other groups studied, with late hatchlings being the youngest to reach these eating activity milestones. This may have been because of the increased stimulus on the late hatchlings by more chicks being engaged in eating-related activities in their surroundings later in the hatching window compared with in the beginning. The early hatched chicks in Paper I showed compensatory growth, since they were lightest at hatch and heaviest at 3 days of age, an effect that has been reported previously in chicks (Lamot *et al*., 2014). The late hatchlings in Paper I were heavier than those in both other hatch groups at placement. According to Tona *et al*. (2003), hatchling weight is linked to egg weight at setting. Moreover, incubation duration increases with egg size (Wilson, 1991), which could explain the higher body weight of the late hatchlings. However, the predictability of post-hatch growth is suggested to be higher when using body weight after access to feed and water, instead of body weight immediate post-hatch, as an indicator (Lindholm *et al*., 2017). The chicks in Paper I were not weighed on the day after first feed intake, but at 3

days of age the body weight of the different groups reflected more accurately the finishing weight of the groups. The early differences in organ weights in Paper I generally did not seem to persist, or to be reflected in the production parameters recorded. Differences in organ development during the first week of life are suggested to be mainly short-term (de Jong *et al*., 2017), as was generally also the case in Paper I.

6.2 Effects of early access to feed and water

Chicks that did not have access to feed and water in the hatcher showed a depressed growth pattern compared with chicks in the positive control group until 25 days of age in Paper II, while some more long-term effects on productivity were observed in Paper III. These results are in line with findings in several other studies of depressed early growth in chicks that are feed-deprived post-hatch (Careghi *et al*., 2005). In Papers II and III, the chicks that were deprived of feed and water were so for 40 h. Feed deprivation has been shown to have negative effects on early growth already at 30 h (Gonzales *et al*., 2003) whereas a 24 h feed deprivation period can be acceptable in terms of maintaining growth and immune function (Juul-Madsen *et al*., 2004). However, in Paper I only 5% of the chicks were involved in eating-related activities at a biological age of 25.4 h, which may explain why effects of feed and water deprivation in chicks after only about 24 h failed to appear (Juul-Madsen *et al*., 2004). Moreover, the adverse effects found by Gonzales *et al*. (2003) after 30 h of feed and water deprivation correspond well with the time when a substantial proportion (50%) of the chicks in Paper I had filled their crop. This indicates that there is a critical turning point at around 30 h when chicks have started to eat and the effects of feed deprivation start to matter and to influence other parameters. Apart from duration of feed and water deprivation, determining the effect of time to first feed intake is essential in understanding the needs of the modern broiler chick and merits further investigation. In retrospect, crop fill assessments would probably have provided valuable information about this if performed at pull in Papers II and III. In Paper III, feed-deprived chicks had lower FI from 11 days onwards than chicks in all other groups except those receiving feed, water and synbiotics in the hatcher and synbiotics in the post-hatch treatment. The combination of feed and water deprivation in the hatcher and provision of synbiotics in the post-hatch

treatment resulted in an inferior FCR at 25 days of age than all other groups, and lower body weight at 32 days compared with all chicks except those in two post-hatch groups. Hence, supplementation with synbiotic had adverse effects when provided after a start in life where chicks lacked feed and water. It is possible this was due to the bacterial strains added raising the maintenance requirements for the birds. Since they were already behind in adding nutrients to their system, the extra load of more 'inhabitants' needing energy may have been too much. This theory was not supported by the microbiota data (see section 6.2.2), but it is important knowledge that bacterial cultures seem not to be exclusively beneficial for the production traits of the host.

6.2.1 Organ development

Early organ development is known to be affected by time to first feed intake (Halevy *et al*., 1999; Bigot *et al*., 2003; Lamot *et al*., 2014), as was also the case in Papers II and III. Feed-deprived chicks displayed lower body weight and YFBM at placement, effects that were mainly due to the maintenance of metabolism still requiring energy even though no exogenous feed was supplied. One risk of feed deprivation is that the chick has to make energy available from its energy stores instead, risking premature depletion of glycogen from liver and muscle deposits (Payne *et al*., 2019). In addition, secretion of yolk to the small intestine has been shown to increase with the presence of feed in the GIT (Noy & Sklan, 2001), which is speculated to be due to solid feed acting as a transportation vessel for yolk mass from the yolk stalk to the intestine. The lack of a transport medium may result in feed-deprived chicks being at risk of utilising yolk at a slower pace, which may negatively affect the uptake of maternal antibodies. However, some studies have found no differences in yolk utilisation between fed and feed-deprived chicks (Bigot *et al*., 2003; Gonzales *et al*., 2003), which is well worth noting and makes it difficult to draw any clear conclusions. Dehydration deriving from time spent in the hatcher without the possibility to hydrate is another possible explanation for the loss of body weight observed in Papers II and III. This suggestion is supported by findings by Fairchild *et al*. (2006) of higher body weight at placement in chicks that were given access to water during feed deprivation for 24 and 48 h at the hatchery.

Early access to feed is a prerequisite for the onset of organ development (Gracia *et al*., 2003), which explains the lower relative weight and length of

the intestines in feed-deprived chicks in Papers II and III. However, in those studies the intestine was weighed and measured as a whole, whereas different segments of the intestine have been shown to be more (duodenum, jejunum) or less (ileum) sensitive to feed deprivation (Geyra *et al*., 2001). Therefore different segments of the intestine should probably be assessed separately in future studies in order to determine more precisely the effects of feed deprivation on specific development of the intestine.

Relative full gizzard weight of feed-deprived chicks at 2 days of age and before feed intake was lower in Paper III, and tended to be lower in Paper II, than that of chicks in the positive control group. However, the same chicks feed-deprived at 2 days of age had greater weight of the empty gizzard compared with fed chicks. It can be speculated that feed-deprived birds give priority to the development of organs higher up in the digestive system, rather than the intestines, when feed access is scarce. In a study by Maiorka *et al*. (2003), relative empty gizzard+proventriculus weight was higher in feed-deprived chicks 48 h post-hatch, which those authors suggested could be due to organs involved in digestion being prioritised in terms of resource allocation in order to better make use of feed when availability is limited. High growth rate has also been found to be correlated to early development of supply organs, rather than demand organs (Christensen, 2009). In addition, in Papers II and III gizzards were weighed in full condition and when emptied. A similar distinction between these two states is not always made in scientific papers, which makes results difficult to compare. Moreover, de Jong *et al.* (2017) concluded from a meta-analysis that there seem to be no long-term effects of feed deprivation on relative weights of heart, liver, gizzard, proventriculus or pancreas, which was also the case in Papers II and III.

6.2.2 Gut microbiota

The microbiota community in Papers II and III seemed to follow the usual development pattern of maturing caeca, where microbial richness and diversity increase with age (Ballou *et al*., 2016). Communal shifts from *Enterobacteriaceae* when young to *Firmicutes* at around one week of age (Ballou *et al*., 2016) are followed by a shift to *Bacteroidetes* at later age (Kubasova *et al*., 2019). The few compositional differences found between hatch treatments in Paper II were no longer apparent at the end of the study. This lack of long-term effects could be due to age-dependent development

of the microbial community, which has been found to be a much stronger driver than post-hatch treatments such as feed deprivation or immediate post-hatch access to feed (Proszkowiec-Weglarz *et al*., 2022).

At the end of the production cycle, there was an associated negative effect on FCR in the group of chicks that were provided with the competitive exclusion (CE) product in water at hatch (Paper II), and also at 25 days of age in the negative control group that received the synbiotic product for 3 days in the research facility (Paper III). However, because there were no compositional differences with regard to microbiota between treatments at later time points in Paper II, and no differences at all in Paper III, it is unlikely that this difference in FCR between the treatments was due to the bacterial cultures added. The provision of beneficial bacteria in different forms (*e.g*. probiotics, CE products, synbiotics) has been suggested to have only small or transient effects on functional properties and activity of the microbial community under non-stressful conditions (Karimi Torshizi *et al*., 2010; Ballou *et al*., 2016). For example, differences have been found in resistance to *Salmonella enterica* in chicks receiving CE products (Schneitz *et al*., 2016).

Differences in microbiota composition have also been suggested to be mainly due to sampling site and, as previously mentioned, age of the bird (Such *et al.*, 2021). In that study, differences were observed when comparing samples collected as ileal mucosa, ileal chymus or caecal chymus. In another study, differences were detected when comparing the results from analyses of luminal and mucosal caecal samples, suggesting that sample type may also have a great influence on microbiota results even if samples are collected in the same region of the GIT (Bindari & Gerber, 2022).

The initial colonisers of the gut have been shown to vary widely in different studies. As pointed out by Richards *et al*. (2019), there is also great variation between studies with regard to the genera that are most abundant in chickens of a particular age. This inconsistency is likely due to chicks being colonised by different genera depending on the hatchery in which they are hatched, management routines *etc*. Later, when the chicks are moved to the rearing farm, they are exposed to a more diverse array of microbes, and microbial diversification and succession in different parts of the gut then accelerates (Richards *et al*., 2019). Because of the great variation between chickens in terms of microbiota composition, the sample size in poultry research has been identified as a possible source of bias because it is

generally quite small, making it difficult to detect differences between treatments (Bindari & Gerber, 2022). There is still much that remains unknown with regard to how the function and structure of microbiota affect intestinal health in chickens. Furthermore, factors that actually characterise a healthy microbiota in poultry are not yet fully understood and need to be further explored (Kers *et al*., 2018).

Immune function

The newly hatched chick is highly dependent on effective transfer of maternal antibodies in order to withstand disease-causing pathogens in its immediate environment in early life (Davison *et al*., 2008). The chick's own synthesis of IgY only begins to occur at around 10 days post-hatch (Härtle *et al*., 2014) and early access to feed and water has been suggested to increase secretion of yolk sac contents (Noy & Sklan, 2001). Moreover, feed-derived antigens and intestinal microbiota have been found to be important for triggering development and maturation of the immune system (Dibner *et al*., 1998; Friedman *et al*., 2003). It was therefore hypothesised in this thesis that early access to feed, water and probiotics during hatch would accelerate the transfer of maternally derived antibodies and give the chicks the necessary prerequisites to initiate endogenous production of IgY more rapidly and/or generate more effective production. This was found not to be the case in Papers II and III, because there were no differences between treatment groups. The increased secretion of yolk sac contents that follows from early feed intake may not mean that transfer of IgY to the chick's circulation is simultaneously and correspondingly increased. For example, Hollemans *et al*. (2021) found no differences with regard to residual yolk between chicks that had early access to feed and chicks that were feed-deprived. To further investigate the activities of IgY uptake in feed-deprived and fed chicks post-hatch, maternally transferred IgY (day 7 post-hatch) in plasma was evaluated, but no differences were found between early feeding strategy treatments (Hollemans *et al*., 2021). It is therefore possible that there are other indicators of (humoral) immune development which may be more suitable for evaluating the effects of early feeding strategies.

Provision of probiotics has been shown to enhance vaccine responses in both meat-type and layer chickens (Kőrösi Molnár *et al*., 2011), while feed deprivation in early life has been found to delay maturation of the adaptive arm of the immune system (Juul-Madsen *et al*., 2004; Bar-Shira *et al*., 2005). In Papers II and III, the response to a novel antigen was assessed by analysing antibody production developed to APV after provision of an inactivated

commercial vaccine toward it. Based on knowledge obtained from previous research, the hypothesis tested in Papers II and III was that early access to feed and water and to probiotics would enhance the immune response to the antigen provided by the APV vaccine. The response to the vaccine was overall very low (as discussed below), which made it difficult to assess treatment-induced differences in responses. Nonetheless, the treatments tested did not induce any evident enhancement of vaccine responders or vaccine-induced antibody production under the circumstances studied. It is evident that the provision of probiotics to enhance vaccine response is far from exclusively effectual. For example, in a review including data on humans comparing the effect of 40 different probiotic strains on the response to 17 different vaccines, favourable effects were found in half of the cases studied (Zimmermann & Curtis, 2018). The reason why probiotics (or other bacterial cultures with beneficial properties) are sometimes favourable and sometimes not seems to depend on different factors such as dose, strain, timing and duration of administration (Zimmermann & Curtis, 2018), as well as administration route (Karimi Torshizi *et al*., 2010). The lack of effect of the bacterial additives on immune function in Papers II and III could thus be due to the chicks being reared under conditions that did not affect their robustness or make them vulnerable to the 'challenge' to which they were subjected. Those conditions included low stocking densities and low pathogen pressure that may have not resembled those in commercial practice. For example, elevated systemic antibody levels have been observed in broiler chickens kept in low sanitary conditions compared with high (Hollemans *et al*., 2021).

In general, the chickens in Papers II and III showed a poor response in terms of total serum levels of IgY, especially from the second sampling onward, and a poor outcome in terms of number of positive responders to the vaccine. In Papers II and III peak IgY in serum was approximately 3.53 mg/mL at day 3 and 0.92 mg/mL at day 31 (see Figure 5). In comparison, Härtle *et al*. (2014) found IgY levels of just below 6 mg/mL at 2 days of age and ~4 mg/mL at 31 days of age, with a lowest recorded value before endogenous production was intensified of just above 2 mg/mL. This illustrates the weakness of the response observed in Papers II and III.

Moreover, in Papers II and III an inactivated vaccine was used, whereas live vaccines are typically used in broiler vaccination programmes because they are believed to be more potent as immune activators (Aida *et al*., 2021). Nevertheless, in some studies, *e.g.* that by Juul-Madsen *et al.* (2004), an inactivated vaccine has been found to provoke a distinct antibody response,

and hence vaccine type is most probably not the only reason for the weak overall response in Papers II and III. The low antibody responses to vaccination observed in Papers II and III led to some adjustment of the experimental design in a later study by our research group (Ivarsson *et al*., 2022). The chicks were kept for a longer period in that study, in an attempt to determine whether giving them more time would increase the number of chicks responding to vaccination. It was found that the longer rearing time gave a slightly higher antibody response, but the percentage of responders was still low (Ivarsson *et al*., 2022). Considering the knowledge acquired from these previous studies, a new approach involving a different (possibly live) vaccine will be employed in future studies in an attempt to generate vaccine responses in a higher proportion of the birds. This will hopefully improve analysis of putative treatment effects on vaccine responses. The low general response to the vaccination made it difficult or even impossible to evaluate the effects of the treatments in Papers II and III.

Supplementation with probiotics with the intention of enhancing vaccine competence and prolonging vaccine protection has been suggested as a relatively cheap strategy lacking major risks. Especially if future research is able to identify the optimal bacterial strains to use in poultry, and the optimal dose and timing of administration in relation to vaccination (Zimmermann & Curtis, 2018). In Paper III (and to some extent in Paper II), however, the chicks that received the bacterial additives and were simultaneously feed-deprived showed poor performance even in later stages of the production cycle, which could be considered a major risk to the producer under commercial conditions.

7. Conclusions

The main finding in this thesis was that feed and water deprivation during hatch resulted in depressed feed intake and corresponding depressed growth in broiler chickens, which in some cases was alleviated during the growing phase. Probiotic supplementation in certain applications had negative effects on productivity, especially in chicks that were also deprived of feed and water during hatch. However, rearing conditions in the experiments were less challenging in terms of stocking densities and pathogen load than those in commercial facilities. Therefore adapted hatching and post-hatch feeding strategies should be further researched in conditions that resemble more closely those encountered by modern commercial broilers of today, so that more comprehensive recommendations can be formulated for the industry. Specific conclusions of the work in this thesis were that:

- On-farmed hatched chicks stayed inactive for a considerable amount of time before engaging in eating-related activities (only 5% were observed performing eating-related activities at a biological age of 25.4 h). Early feeding strategies may therefore not be of benefit to the average on-farm hatched chick compared to its conventional counterpart if only productivity is taken into account when hatchery and farm are at a reasonable distance (short transport time).

- Early hatchlings had compensated for their lower hatching weight already by 3 days of age, when they were the heaviest group. Early differences in organ parameters generally levelled out throughout the study.

- Post-hatch feed and water deprivation in the hatcher resulted in reduced weight gain and feed intake. Some of the early differences observed in

productivity and most of the early differences in organ parameters had disappeared by the end of the study.

- Addition of probiotics in combination with post-hatch feed and water deprivation amplified the adverse effects of the latter, generating long-term effects on production parameters under the experimental conditions. Therefore provision of probiotics to chicks that are also starting their life without access to feed and water cannot be recommended without further investigation.

- Different immune traits analysed were unaffected by hatch and post-hatch treatment. Likewise, no long-term effects on microbiota composition were found. However, the outcomes for these parameters could differ if they are evaluated in an environment more closely resembling that in commercial production.

8. Practical implications and future perspectives

In Sweden, where hatchery and rearing farm are located relatively close together geographically, on-farm hatching is probably not of benefit to relieve negative effects of feed and water deprivation in the average chick. This due to the observed long time to first feed intake in newly hatched chicks combined with a condensed hatching window and short transportation time. On-farm hatching is labour-intense initially and requires a more knowledgeable producer, and it also brings a considerably higher risk that is carried only by the individual chicken producer. As was the case for the on-farm hatched chicks, the average hatchery-hatched chick may not benefit from provision of feed and water in the hatchery. However, there will always be chicks that are not average, and there will sometimes be hold-ups during transportation or processing of chicks at the hatchery for different reasons that are beyond the operator's control. Therefore, when breeding animals, the precautionary principle should probably be applied in order to guarantee that *e.g*. the 'freedom from hunger and thirst' criterion in the 'The Five Freedoms' standard of animal welfare can be met.

Moreover, while reduced weight gain and organ development in early life may not cause any problems if downstream conditions are good, if circumstances such as high pathogen load or other obstacles arise it will always be advantageous to have some flexibility in terms of extra resilience and robustness in the chicks. Furthermore, replacing conventional hatchers and transportation vehicles with types that permit feed and water provision (or transport of viable eggs for on-farm hatching) is a major investment for the hatchery industry and producer that would ultimately have to be compensated for by consumer willingness to pay more for chicken meat produced in that way. Knowledge among the general public of how animal

products are produced today is low, and most consumers of chicken meat are probably not aware that chicks are provided with feed and water only on reaching the rearing farm.

There is intensive ongoing research on development of bacterial cultures for provision to poultry and other livestock animals. Products engineered for specific purposes, such as particular ages, environments or challenges, will likely reach the market in the near future, so the opportunity for providing such additives already in the hatchery should not be dismissed. More research is needed on other parameters that affect the chick's start in life at the hatchery (*e.g*. light, noise, dust, pathogen load, disinfectants *etc*.). In addition, the welfare status of conventionally hatched chicks compared with on-farm or hatchery-fed chicks should be further investigated. Finally, newly developed hatching concepts that allow the chicks greater freedom in early life need to be tested further and more broadly under conditions more similar to those in commercial settings, thereby including actual real-life challenges that the chicks commonly encounter, in order to formulate more substantiated recommendations for the industry.

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Populärvetenskaplig sammanfattning

Den svenska kycklingen har kommit att bli en favorit på matbordet och sedan 80-talet har konsumtionen fyrdubblats till 23.1 kg kycklingkött per person och år. Kycklingen, som kläcks på ett kläckeri och senare ska komma att växa upp på en gård hos en kycklingbonde, har från ägget med sig en matsäck, gulesäcken, som dras in i kycklingens mage strax före kläckning. Näringen i gulesäcken är viktig eftersom det inte finns möjlighet att i traditionella kläckare förse kycklingen med foder och vatten. En tidigt kläckt kyckling kommer att behöva vistas i kläckaren till dess att alla syskon är kläckta, vilket kan ta upp till 48h. Utöver detta så kallade kläckningsfönster, tar det dessutom lite tid att gå igenom kycklingarna och se att de håller en god kvalitet och sedan transportera dem till gården. I Sverige tar det ungefär 25 timmar för en kyckling kläckt mitt i kläckningsfönstret att få tillgång till foder och vatten ute på gården. Tidig tillväxt har i studier visat sig påverkas redan vid 30 timmars brist på foder och vatten, medan man sett att immunförsvaret påverkas vid 48 timmar men inte vid 24 timmars brist. Förutom näring som kycklingen behöver under sina första dagar innehåller gulesäcken också viktiga antikroppar. Den nykläckta kycklingens immunförsvar är outvecklat och därför är antikropparna, som kycklingen fått via ägget, livsviktiga för att skydda den mot bakterier och virus som annars hade kunnat göra den sjuk.

Den kyckling vi äter idag har förändrats mycket de senaste decennierna, mestadels på grund av att man avlat på djur med hög tillväxt och på så sätt fått kycklingar som växer snabbt och är mycket effektiva på att omvandla foder till muskler. Det finns en misstanke om att gulesäcken hos dagens betydligt mer snabbväxande kyckling kanske inte är tillräcklig för att stötta den med den näring och de antikroppar den behöver, och därför har nya system och ny utrustning utvecklats för att minska tiden mellan kläckning

och första utfodringstillfälle. Exempel på detta är gårdskläckning, där de befruktade äggen transporteras till gården efter 18 dagars inkubation och får kläckas hos kycklingbonden, och modernare kläckare som ger kycklingarna möjlighet till foder- och vattentillgång under pågående kläckning.

Den här doktorsavhandlingen bygger på två olika studier. Den första syftade till att undersöka hur tiden från kläckning till första foder- och vattenintag påverkade tillväxt och organutveckling. Detta undersöktes hos kycklingar som fick kläckas direkt i försöksanläggningen. Vi studerade hur lång tid det tog innan kycklingarna började utföra födosöksrelaterade beteenden samt hur lång tid de tog för kycklingarna att fylla sin kräva. Krävan är en liten utbuktning på kycklingens matstrupe där den lagrar foder innan det transporteras vidare ned i matspjälkningssystemet.

Eftersom kläckningstiden registrerades och kycklingarna delades in i tre kläckningsgrupper (tidigt kläckta, medelkläckta och sent kläckta) kunde vi utvärdera hur effekterna av placering i kläckfönstret påverkade olika produktivitetsmått som är allmänt använda inom produktionen (tillväxt, foderintag och foderomvandlingsförmåga), tid till första foderintag, krävfyllnad, utveckling av organ samt enzymer som används vid matspjälkningen. Vi fann att kycklingarna tog lång tid på sig att påbörja födosökande beteenden och även att börja äta. Vissa fysiologiska skillnader fanns mellan de olika kläckgruppernas kycklingar tidigt i livet, men de tenderade att försvinna med tiden. De tidigt kläckta kycklingarna vägde minst vid försökets början, men visade prov på kompensatorisk tillväxt då de var den tyngsta gruppen vid tre dagars ålder.

I den andra studien ville vi undersöka vilka effekter tillgången till foder, vatten samt probiotiskt tillskott redan i kläckaren kunde ha på produktivitet, organutveckling, mikrobiotans sammansättning i kycklingens blindtarm samt effekter på vissa av immunförsvarets egenskaper. (Probiotika kallas de preparat innehållande levande mikroorganismer som när de administreras i tillräcklig mängd har hälsofrämjande effekter på den som intar dem.) Vi kunde se att kycklingar som fick vara utan foder och vatten i kläckaren åt mindre och växte sämre under tillväxtfasen (i försöksstallet). Tillskott av probiotika visade sig däremot generera vissa negativa långtidseffekter på produktiviteten, och extra tydlig blev den effekten när det gavs till de kycklingar som under kläckningen samtidigt var utan tillgång till foder och vatten. Inga långtidseffekter av tidig utfodring i kläckaren eller tillskott av

probiotika stod heller att finna med avseende på mikrobiotans sammansättning eller de immunparametrar som analyserades.

Sammanfattningsvis observerade vi att kycklingarna i gårdskläckningsförsöket börja äta så pass sent efter kläckning, att den tidigare tillgången till foder och vatten kan antas spela liten roll för den genomsnittliga nykläckta kycklingen när kläckeriet ligger geografiskt nära gården. Samtidigt som gårdskläckning kräver stora arbetsinsatser vid kläckning kräver det också mer av den enskilda kycklingbonden i form av kunskap och den risk som en enskild kycklingföretagare tar på sig i form av investeringar i det nya systemet. Däremot kommer det alltid att kläckas kycklingar som inte är genomsnittliga avseende kläckningstidpunkt, och för dessa kycklingar kan tidig tillgång till mat och vatten ändå vara värdefull. Det kan också uppstå situationer utanför vår kontroll där till exempel kycklingar blir kvar på kläckeriet längre än planerat på grund av exempelvis problem med transporten eller andra yttre faktorer. Det kan därför vara en god idé att tillämpa försiktighetsprincipen när man hanterar stora mängder djur, för att kunna säkerställa att de inte går hungriga eller törstiga, vilket är ett av kriterierna i välfärdsstandarden De fem friheterna.

Kycklingarna i den här doktorsavhandlingen har visat att de åtminstone delvis är förmögna att kompensera för de negativa effekter som fördröjd tillgång till foder och vatten har på både produktivitet och organutveckling, men hade det varit lika självklart att de hade kunnat göra det under tuffare förhållanden? Förmodligen är det klokt att ta lite höjd för oförutsedda utmaningar såsom högt smittryck genom att se till att det är extra robusta och ”startklara” kycklingar som kommer till kycklingbonden.

Vidare hade utbytet av traditionella kläckare och transportfordon, till fördel för moderna kläckare med kapacitet att förse kycklingarna med foder och vatten, varit en investering för kläckindustrin och kycklingbönderna. I slutändan kräver kostnaderna för dessa investeringar att konsumenterna är villiga att betala mer för kycklingkött som producerats på detta sätt. Då krävs också att konsumenten har den kunskap som krävs för att kunna utvärdera för- och nackdelar med de olika koncepten, vilken förmodligen saknas idag.

Det forskas intensivt kring probiotiska tillskott till livsmedelsproducerande djur, och förhoppningsvis ser vi snart produkter som är riktade mot specifika ändamål, så som känsliga åldrar och särskilda miljöer eller utmaningar, och som kan hjälpa kycklingarna till en mer flygande start i livet. Mer forskning bör också riktas mot andra parametrar

som kan påverka kycklingens första tid i livet på kläckeri, såsom till exempel ljud, brist på ljus, damm, smittryck, hantering och användning av desinfektionsmedel. Välfärdsaspekten (till exempel strökvalitet, förekomst av hälta, fjäderdräktens renlighet) hos konventionellt kläckta kycklingar jämfört med gårdskläckta och kläckeriutfodrade kycklingar bör också undersökas närmare.

Något som bör tas i beaktande är att studierna i doktorsavhandlingen genomfördes i en kontrollerad försöksanläggning där beläggningsgrad och smittryck var betydligt lägre än i kommersiella anläggningar, något som kan ha kommit att påverka resultaten. Därför bör de nya kläckningskoncepten som möjliggör en högre grad av frihet för kycklingen tidigt i livet också testas vidare och bredare under förutsättningar liknande de som gäller vid kommersiell produktion, där vanliga utmaningar som kycklingarna stöter på genom livet ska finnas representerade. Detta för att kunna föreslå mer välgrundade rekommendationer till kycklingnäringen.

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Effect of hatching time on time to first feed intake, organ development, enzymatic activity and growth in broiler chicks hatched on-farm

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ABSTRACT

The conventional commercial hatcheries used today do not allow the newly hatched chicks to consume feed or water. Combined with natural variation in hatching time, this can lead to early hatched chicks being feed-deprived for up to 72 h before being unloaded at the rearing site. This study investigated the effects of hatching time on time to first feed intake and development of organs, digestive enzymes and productivity in terms of growth and feed conversion ratio in chicks hatched on-farm. Chicks were divided into three hatching groups (early, mid-term and late), and assessed over a full production cycle of 34 days. The results revealed that chicks remain inactive for a considerable amount of time before engaging in eating-related activities. Eating activity of 5% (i.e. when 5% of birds in each hatching group were eating or standing close to the feeder) was recorded at an average biological age (**BA**) of 25.4 h and a proportion of 50% birds with full crop was reached at an average BA of 30.6 h. Considering that the hatching window was 35 h in this study, the average chick probably did not benefit from access to feed and water immediately post-hatch in this case. At hatch, mid-term hatchlings had a heavier small intestine (30.1 g/kg bw) than both early (26.4 g/kg bw) and late (26.0 g/kg bw) hatchlings. Relative length of the small intestine was shorter in late hatchlings (735 cm/kg bw) than in mid-term (849 cm/kg bw) and early (831 cm/kg bw) hatchlings. However, the relative weight of the bursa fabricii was greater in mid-term (1.30 g/kg bw) than in early hatchlings (1.01 g/kg bw). At hatch, late hatchlings were heavier than early and mid-term hatchlings ($P < 0.05$), but by 3 days of age early hatchlings were heavier than mid-term and late hatchlings ($P < 0.01$). The only effect persisting throughout the study was a difference in the relative weight of the small intestine, where late hatchlings had heavier intestines than early hatchlings ($P < 0.05$). Thus, while there were differences between hatching groups, this study showed that the hatchlings seemed capable of compensating for these as they grew.

Implications

Prolonged time without access to feed and water has been proven to have negative effects on the subsequent growth of broiler chickens, but the time it takes for a newly hatched chick to engage in eating-related activities has not been determined. This study found that newly hatched chicks rest for a considerable time before seeking feed and water. This finding is important when planning studies focusing on chicks' early life. It is also relevant for the chicken industry when considering which management system to invest in (i.e. on-farm hatching systems).

Introduction

The conventional way of hatching broiler chickens may not be optimal from a biological point of view. Even though all eggs are put into the hatcher at the same time at the hatchery, the chicks hatch over a period depending on the biological variation and egg storage time. This period is often referred to as the hatching window and according to Tong et al. (2013) it ranges from 24 to 48 h. Powell et al. (2016) observed a hatching window of 37 h for Ross 308 chickens. Even though there are new hatching concepts in use allowing provision of feed and water for the newly hatched chick (Van der Pol et al., 2015) the transition to such hatchery practices has started only during the last couple of years. Therefore, during commercial conditions, a broad hatching window will increase the time to first feed and water intake at the rearing site. At pull, when the majority of the chicks have hatched, management routines at the hatchery and loading and transportation add to the delay (Van de Ven et al., 2013). According to Willemsen et al. (2010), some chicks may be feed-deprived for up to 72 h on arrival at the rearing site. Although the residual yolk supports the chick with nutrients immediately post-hatch (Noy and Sklan, 2001), delayed access to feed and water has been shown to have adverse effects on early chick growth (Noy and Sklan, 1999; Sklan et al., 2000), muscle cell proliferation

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(Powell et al., 2016), yolk sac utilisation (Noy and Sklan, 2001), development of the gastrointestinal tract (Lamot et al., 2014) and immune function (Bar Shira et al., 2004).

At hatch, the chick's digestive system has to undergo considerable changes to convert to digestion of exogenous feed rich in carbohydrates, instead of the lipids that constitute the majority of the yolk (Uni et al., 1998; Ravindran, 2003). The chick actually begins preparing for ingestion of exogenous feed during the neonatal state. During embryonic development, the pancreas starts to secrete digestive enzymes to the neonate chick's intestine. However, the digestibility of starch is low at hatch and increases with age (Marchaim and Kulka, 1967; Noy and Sklan, 1998; Ravindran, 2003).

To overcome the possible disadvantages of deprivation of feed and water post-hatch, different concepts for on-farm hatching have been developed in the Netherlands, where brooded eggs are transported from the hatchery to the rearing facilities at embryonic day 18. Chicks are then hatched during embryonic days 20–21 and provided with immediate access to feed and water (Van de Ven et al., 2009). Chicks hatched at different times during the hatching window have been shown to differ physiologically at hatch in e.g. organ weight, yolk uptake (Van de Ven et al., 2013) and feeding behaviour (Nielsen et al., 2010). However, to the best of our knowledge, no previous study has investigated whether these differences persist and are significant later in the growing period. The aim of the present study was thus to evaluate the effects of hatching time on time to first feed intake and development as regards organ size, secretion of digestive enzymes and growth in chicks hatched on-farm, in a trial ending at 34 days of age.

Material and methods

Housing, birds and feed

The eggs, laid by a 40-week-old breeder flock, had been stored for 4 days prior to incubation and were incubated at 37.8 °C at the commercial hatchery SweHatch, Väderstad. At embryonic day 17.5, 400 Ross-308 eggs that had been automatically candled to confirm fertilisation were transported 309 km by car (approximately 3.5 h) to Lövsta Research Centre at Uppsala, Sweden. The eggshell temperature was checked regularly during transportation using an ear thermometer (Braun ThermoScan® 5, Braun GmbH, Kronberg, Germany).

When the eggs were placed at the research centre, the temperature in the animal facility was set to 33 °C for the first 3 days and was thereafter successively lowered until it reached 23 °C at 24 days and throughout the study. The relative humidity was around 40%. During the period of hatch at the animal facility, starting at embryonic day 19, the first third of the chicks to hatch were assigned to an 'early' hatching group ($n = 95$), the second third to a 'mid-term' hatching group ($n = 95$) and the remaining chicks to a 'late' hatching group ($n = 95$). Day 0 was defined as the day the peak of the hatching window took place, namely embryonic day 20. As soon as the feathers of a chick had dried, it was weighed and placed in one of five replicate modules assigned to the relevant hatching group. There were 15 modules in total.

Each module measured 1.5 m × 0.75 m and contained a feeder and three nipple drinkers to which the chicks had immediate access post-hatch. When the experiment started, there were 16.9 chicks/m^2, whereas the Swedish regulations allow a maximum of 25 chicks/m^2 (Swedish Board of Agriculture, 2019). At the end of the study the stocking density was 16 kg/m^2, whereas the maximum density according to European Union (**EU**) regulation is 33 kg which can be expanded to 39 or even 42 kg/m^2 if certain criteria are fulfilled (Council of the European Union, 2007). Wood shavings were used as litter material. Constant light was provided during hatch and for 2 days post-hatch. On day 3, the chicks were given 1 h of darkness between 23.00 h and midnight. Thereafter, the chicks were provided with 1 h of extra darkness per night until day 8. From day 8 until the end of the study, lights were off between 23.00 and 05.00 h. In the first days, chick body temperature was determined regularly following the recommendations given in the Ross broiler handbook (Aviagen, 2018) by recording the vent temperature of the chicks using the ear thermometer (Braun ThermoScan® 5, Braun GmbH, Kronberg, Germany). A body temperature of 39.4–40.5 was considered optimal (Aviagen, 2018).

The chicks were fed crumbled, sieved pellets as a starter feed and then switched to a grower feed at 10 days of age (both feeds Svenska Foder AB, Lidköping). All birds were given the same commercial starter and grower feeds (pellet diameter 3.5 mm). No coccidiostats were used. Feed samples were dried at 103 °C for 16 h for analysis of DM, while ash was analysed after incineration for 3 h at 550 °C (Jennische and Larsson, 1990). Crude protein (**CP**) content (N × 6.25) was determined by the Kjeldahl method (Nordic Committee on Food Analysis, 2003). Ether extract was determined according to the European Communities (EC) (1998). The analysed chemical composition of the feed was (g/kg DM): ash 57, CP 243, crude fibre 33 and ether extract 53 in the starter feed and ash 48, CP 229, crude fibre 43 and ether extract 64 in the grower feed. The calculated energy content (according to EU MJ) was 13.6 AME MJ/kg DM for the starter feed and 14.5 AME MJ/kg DM for the grower feed.

Recordings

See Table 1 for the number of chicks used at every sampling occasion.

Chick length and organ development

At hatch, live weight and chick length (from middle toe to beak in chicks placed belly down, measured by the same person) were recorded for 20 chicks per hatching group. These chicks were then euthanised by neck dislocation and dissected to determine the weight of the yolk sac, small intestine (with intestinal content), bursa fabricii, heart, liver, gizzard (as dissected and also after emptying and washing) and proventriculus (as dissected), and length of small intestine. At 6, 10, 20 and 34 days of age, two birds from each replicate module were euthanised (by a blow to the head followed by neck dislocation in young chicks and by an intravenous injection of pentobarbital sodium, 100 mg/ml, in chicks aged 20 and 34 days) and weight and length of organs were determined. Thus, at hatch there were 95 birds per hatching treatment (i.e. 19 chicks per module), whilst at the end of the study only 40 chicks per hatching treatment remained (i.e. 8 chicks per module) because of reduction of the number of birds due to both sampling and evening of groups (25 birds per hatching group were excluded from the experiment at day 10) (Table 1).

Enzymatic activity

Samples from the pancreas and small intestine were collected from every second bird used for organ sampling. The duodenal loop was identified and an approximately 5 cm tissue sample starting from the apex, including both intestine and pancreas, was taken and immediately frozen at −80 °C for later analysis of enzymatic activity.

For α-amylase activity assays, intestinal and pancreatic samples from days 6, 10, 20 and 34 were thawed separately, washed with ice-cold phosphate buffer saline, individually cut into small pieces and suspended in 20 volumes of ice-cold malic acid buffer (pH 5.4) and homogenised in an electrical homogeniser (Ultra turrax tube dispenser, IKA Werke GMBH & Co.KG, Staufen, Germany). The homogenate was centrifuged for 10 min at 15 800 × *g* and aliquots of the supernatant were stored at −80 °C for later analysis. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF; 0.5 mM; Sigma no. P7626, Sigma–Aldrich Sweden AB, Stockholm, Sweden) was added before the homogenate was analysed for α-amylase activity. For day 0 samples, intestine plus early pancreas tissue were homogenised together, due to

Table 1
Number of chickens euthanised and used in different recordings at hatch, day 6, 10, 20 and 34. Remaining number of chicks per hatching group (HG) after each sampling occasion is also presented.

Recording	At hatch	Day 6	Day 10	Day 20	Day 34
Organ weights and lengths	20 chicks per HG	2 birds per replicate module, i.e. 10 chicks per HG[1]	2 birds per replicate module, i.e. 10 chicks per HG	2 birds per replicate module, i.e. 10 chicks per HG	2 birds per replicate module, i.e. 10 chicks per HG
Small intestine measurements	10 chicks per HG	1 bird per replicate module, i.e. 5 chicks per HG	1 bird per replicate module, i.e. 5 chicks per HG	1 bird per replicate module, i.e. 5 chicks per HG	1 bird per replicate module, i.e. 5 chicks per HG
Amylase activity	5 chicks per HG	1 bird per replicate module, i.e. 5 chicks per HG	1 bird per replicate module, i.e. 5 chicks per HG	1 bird per replicate module, i.e. 5 chicks per HG	1 bird per replicate module, i.e. 5 chicks per HG
Evening of groups			5 birds per replicate module, i.e. 25 chicks per HG		
Remaining chicks per hatching group after sampling	95	85	50	40	0[1]

[1] The experiment was ended.

lack of material. Apart from this, the procedure was the same for all samples.

Level of α-amylase activity was determined with a commercial kit (Ceralpha Method, AOAC Official Method 2002.01., Megazyme International Ireland Ltd., Wicklow, Ireland) using benzylidene end-blocked *p*-nitrophenyl-α-D-maltoheptaoside as substrate. Test tubes containing the homogenate and amylase high-range reagent solution were incubated for 20 min at room temperature. The reaction was stopped by adding stopping solution (alkaline solution) and absorbance was recorded at 405 nm.

Crop fill assessment

Approximately 4 h after completion of hatchling placement, four randomly pre-selected focal chicks in each module were gently picked up and their crop was examined by palpation (Aviagen, 2018) to determine whether it was empty, half-full or full. This was done every 4 h for 36 h.

Behaviour observations

Behaviour observations commenced within 3 h after completed placement in the early hatching group and were extended to the two later hatching groups after completed placement of chicks in those groups. Chick behaviour was studied by scan sampling every hour, to determine time to first feed intake. Three persons in total took terms quietly walking down the stable aisles, recording the observed behaviours. These persons had beforehand synchronised the methodology to be able to perform the scan sampling as equal as possible. The number of chicks performing either of the following behaviours was recorded: (a) eating from the feeder while standing on the floor or on the feeder or (b) standing close to the feeder or on top of the feeder. A maximum distance of 5 cm from the feeder was considered close. Behaviour observations continued for 52 h.

Production performance

Bird live weight and feed intake were recorded weekly for each replicate module. All chicks in each module were weighed together in a basket placed on a scale outside the module. Weights were then divided by the number of chicks in the module at the weighing occasion. Feed conversion ratio (kg feed consumed divided by kg growth, i.e. FCR) was calculated from these results. Mortality was recorded daily.

Statistics

Growth, digestive enzyme and organ weight data were analysed using the Procedure Mixed (PROC MIXED), in the statistical program SAS (version 9.4), with hatching treatment and age as fixed factors and module as a random factor. The behaviours 'eating' and 'standing close to the feeder' were combined and defined as 'eating activity'. The proportion of chicks performing eating activity was analysed with the statistical software R, using a mixed logistic regression model with module as a random effect and a smooth spline component with respect to time from observation start. The model was used to estimate proportions with 95% confidence intervals and test differences in eating activity with respect to biological age (**BA**), defined for each hatching group as time elapsed in hours since hatch of the median chick in that group. Crop fill measurements were analysed with the statistical software R, using a mixed ordinal regression model assuming proportional odds, with module as random effect and observation time as a categorical variable. The model was used to estimate odds ratios (**OR**) with respect to BA, where a ratio > 1 indicates higher probability of the numerator (first-mentioned factor) than the denominator (second factor), and a ratio < 1 the reverse.

For growth, FCR, eating activity and crop fill, module was considered the experimental unit, giving five replicates per hatching treatment. Organ development and enzyme activity was analysed with individual animal as experimental unit.

Results

The first third of chicks (early hatching group) hatched within 476–496 h post start of incubation, the second third (mid-term group) hatched within 496–504 h and the remaining third (late group) within 505–511 h. The length of the hatching window for all chicks hatched was thus 35 h.

Body weight and organ development at hatch

Data collected from a sample of chicks ($n = 20$ per hatching group) immediately after hatch showed that there were no differences between hatching groups with regard to BW, yolk-free body mass (**YFBM**), yolk sac, chick length, heart, liver, gizzard and proventriculus weighed together, or gizzard alone (as dissected) (Table 2). However, early and late hatchlings had a lighter small intestine at hatch than mid-term hatchlings (Table 2). Length-wise, at hatch late hatchlings had a shorter small intestine in relation to BW than both early and mid-term hatchlings (Table 2). Moreover, there was a difference in bursal weight between hatching groups, with the mid-term group having relatively heavier bursa fabricii than the early hatching group.

Growth, feed conversion ratio and organ development during the growing period

There were no differences in FCR between hatching groups throughout the experimental period (Table 3). However, there was a difference in BW between hatching groups at 0 and 3 days of age. At hatch, late hatchlings were heavier than both early and mid-term hatched chicks, but by 3 days of age the early hatchlings were heavier than both

Table 2
Body, yolk sac and organ weight at hatch in chicks hatched early, mid-term and late in the hatching window. Organ weights are expressed as a proportion of BW.

Variable	Hatching group			Pooled SEM	*P*-value
	Early [1]$n = 20$ [2]$n = 10$	Mid-term [1]$n = 20$ [2]$n = 10$	Late [1]$n = 20$ [2]$n = 10$		Hatching group
BW (g)	[3]43.53	43.97	45.34	0.40	ns
Yolk sac (g)	6.22	5.95	6.86	0.16	ns
YFBM[5] (g)	37.3	38.0	38.5	0.36	ns
Chick length (cm)	18.4	18.8	18.5	0.07	ns
Yolk sac (g/kg BW)	142.5	134.9	151.9	3.39	ns
Small intestine[2] (g/kg BW)	[4]26.4[b]	30.1[a]	26.0[b]	0.65	*
Small intestine[2] (cm/kg BW)	831[a]	849[a]	735[b]	14.7	**
Bursa fabricii (g/kg BW)	1.01[b]	1.30[a]	1.14[ab]	0.05	*
Heart[2] (g/kg BW)	8.51	8.80	8.59	0.18	ns
Liver[2] (g/kg BW)	20.3	21.5	18.8	0.43	ns
Proventriculus and gizzard[2] (g/kg BW)	45.2	47.8	44.9	0.90	ns
Gizzard[2] (g/kg BW)	38.4	39.0	37.6	0.73	ns

**$P < 0.01$; *$P < 0.05$; ns $P > 0.05$.
[1] A total of 20 chicks from each hatching group were euthanised and weight, length, yolk sac weight and bursal weight were recorded for all birds.
[2] Small intestine, heart, liver, proventriculus and gizzard measurements were performed on every second bird.
[3] Values are least-squares means (LSM).
[4] LSM values within rows lacking a common superscript are significantly different ($P < 0.05$).
[5] Yolk free body mass (YFBM).

mid-term and late hatchlings. The early hatching chicks had numerically greater weight throughout the study, but from 10 days of age there were no significant differences in BW (Table 3).

As regards weight of organs during the growing period (Table 4), the small intestine was the only parameter differing between hatching groups, with early hatched chicks having significantly lower relative intestinal weight than late hatchlings and a tendency for lower intestinal weights than mid-term hatchlings ($P = 0.0747$, data not shown). At 20 days of age, the yolk sac was completely utilised and not detectable during dissection.

An effect of age was also observed for all organs studied (Table 4). Weight or size decreased with age when considered as a proportion of the total BW for yolk sac, small intestine (g and cm), heart, liver, proventriculus and gizzard. The relative weight of the bursa fabricii was significantly greater at 20 days of age than at 6, 10 and 34 days of age. At 34 days of age, early hatchings were heavier than both mid-term and late hatching chicks, resulting in an interaction between hatching group and age.

Table 3
Feed conversion ratio – g/g growth (FCR) and BW – g (BW) at seven different ages (days) in chicks hatched early, mid-term and late in the hatching window.

	Hatching group			Pooled SEM	*P*-value
	Early $n = 5$	Mid-term $n = 5$	Late $n = 5$		
FCR					
Day 0	–	–	–	–	–
Day 3	[1]0.93	0.97	1.00	0.018	ns
Day 10	1.09	1.09	1.06	0.011	ns
Day 17	1.61	1.63	1.59	0.030	ns
Day 24	1.52	1.54	1.55	0.024	ns
Day 31	1.51	1.60	1.52	0.031	ns
Day 34	1.56	1.57	1.56	0.008	ns
BW					
Day 0	[2]44.8[b]	44.9[b]	46.3[a]	0.19	*
Day 3	77.1[a]	67.9[b]	64.3[b]	1.11	**
Day 10	307.1	286.0	278.7	10.09	ns
Day 17	718.3	651.2	655.9	23.80	ns
Day 24	1 273.5	1 221.7	1 158.9	32.14	ns
Day 31	1 928.7	1 889.7	1 826.2	48.65	ns
Day 34	2 232.9	2 190.3	2 136.3	56.50	ns

**$P < 0.01$; *$P < 0.05$; ns $P > 0.05$.
[1] Values are least-squares means (LSM).
[2] LSM values within rows lacking a common superscript differ ($P < 0.05$).

Enzymes at hatch and during the growing period

No effect of hatching group on α-amylase activity (U/g sample, where U is μmol hydrolysed per minute) was observed when analysing mixed or separated intestinal and pancreatic samples, either at hatch or later in the study (Table 5).

Intestinal α-amylase in relation to intestinal content was higher at 6 days of age than at 20 and 34 days of age (Table 5). Moreover, there was an interaction between hatching group and age with regard to α-amylase (U/g sample) activity in the intestine. This interaction arose because there was no effect of age within the early and late hatching groups, whereas the mid-term chicks had higher α-amylase activity in the intestine at 6 days of age compared with 10, 20 and 34 days of age.

In the pancreas, α-amylase activity (U/g sample) was lower at 6 and 10 days of age than at 20 and 34 days (Table 5).

Eating activity

The percentage of chicks showing active eating behaviour and related confidence intervals at BA 20, 30, 35 and 40 h is shown in Table 6. There were differences between hatching groups in their eating activity in relation to BA. Comparisons of confidence intervals between hatching groups (Table 6) revealed that eating activity was higher in the late hatching group than in the early hatching group at BA 20. There was also a tendency for a difference ($P = 0.062$, data not shown) between the late and mid-term hatching groups at the same BA. At BA 30, eating-related activity was highest in the mid-term group and lowest in the early group, whereas the late group was intermediate and not different from either the early or mid-term group. At BA 35 and 40, eating activity was higher in the early group and mid-term group compared with the late group, but there were no differences between the early and mid-term groups. An eating activity level of 5% (i.e. when 5% of the birds were either eating or standing close to the feeder) was reached at BA 21.7 h in the late hatching group, 25.1 h in the mid-term hatching group and 29.5 h in the early hatching group, hence, a 5% eating activity was observed first at a mean BA of 25.4 h (data not shown).

Table 4
Organ weight (as proportion of BW) at four different ages in chicks hatched early, mid-term and late in the hatching window. Values for hatching groups are averages for the 34 days growth period.

Variable	Hatching group			Age (days)				Pooled SEM	*P*-value		
	Early $n = 40$ [1]$n = 20$	Mid-term $n = 40$ [1]$n = 20$	Late $n = 40$ [1]$n = 20$	6 $n = 30$ [1]$n = 15$	10 $n = 30$ [1]$n = 15$	20 $n = 30$ [1]$n = 15$	34 $n = 30$ [1]$n = 15$		Hatching group	Age	Hatching group * age
Weight (g)	[2]955.4	862.7	863.8	143.3[d]	294.8[c]	890.7[b]	2 246.9[a]	21.2	ns	***	*
YFBM[4] (g)	231.4	214.4	210.9	143.1[b]	294.8[a]			6.8	ns	***	ns
Yolk sac (g/kg BW)	0.63	0.72	0.76	1.31[a]	0.11[b]			0.13	ns	***	ns
Small intestine[1] (g/kg BW)	[3]77.5[b]	84.2[ab]	85.4[a]	105.4[a]	90.2[b]	76.1[c]	57.8[d]	1.22	*	***	ns
Small intestine[1] (cm/kg BW)	286.5	341.1	341.0	678.6[a]	395.8[b]	152.3[c]	64.8[d]	14.8	ns	***	ns
Bursa fabricii (g/kg BW)	1.68	1.78	1.68	1.44[b]	1.66[b]	2.05[a]	1.71[b]	0.04	ns	***	ns
Heart (g/kg BW)	7.06	6.85	7.04	8.64[a]	8.07[a]	6.05[b]	5.17[b]	0.17	ns	***	ns
Liver (g/kg BW)	38.7	38.6	41.2	50.1[a]	44.3[b]	34.5[c]	29.1[d]	0.55	ns	***	ns
Proventriculus (g/kg BW)	41.6	43.7	42.1	72.7[a]	46.3[b]	32.2[c]	18.5[d]	1.05	ns	***	ns
Gizzard full (g/kg BW)	33.4	35.5	33.5	61.2[a]	37.0[b]	24.8[c]	13.6[d]	0.88	ns	***	ns
Gizzard empty (g/kg BW)	21.4	23.2	22.4	36.1[a]	25.4[b]	17.8[c]	10.1[d]	0.71	ns	***	ns

***$P < 0.001$; *$P < 0.05$; ns $P > 0.05$.
[1] Small intestine measurements were performed on every second bird.
[2] Values are least-squares means (LSM).
[3] LSM values within rows lacking a common superscript differ ($P < 0.05$).
[4] Yolk free body mass (YFBM).

Crop fill

As illustrated in Fig. 1, the BA at which all four focal birds (100%) from each module had either half-full or full crop differed between hatching groups, decreasing from 40.6 h in the early hatching group to 32.4 h in the mid-term hatching group and 30.5 h in the late hatching group. This indicates that chicks in late and mid-term groups started to eat earlier post-hatch than the early hatching chicks. The three hatching groups reached a proportion of 50% of birds with full crop at approximately the same BA (32.6, 28.6 and 30.5 h in the early, mid-term and late hatching group, respectively) (Fig. 1). According to OR the mid-term group had a higher proportion of full crops than the early group at BA 30 h (OR = 6.3) and 35 h (OR = 4.5) (Table 7). A similar pattern was seen at BA 35 h for the mid-term hatching group compared with the late hatching chicks (OR = 3.6), while a tendency for an effect was observed at BA 40 h (OR = 3.0). At BA 40 h, the early hatching group tended to have a higher proportion of full crops than the late group (OR = 0.3) (Table 7). The increase from a proportion of 50% birds with full crop to 90% took an extra 7 h for the early hatching group and 3 h for the mid-term group. In the late group, only 65% of the birds had a full crop at BA 43 h, when the measurements ended (Fig. 1).

Table 6
Proportion of chicks active in eating-related behaviours and confidence intervals between chicks hatched early, mid-term and late in the hatching window. Biological age (BA) is defined for each hatching group as time (h) since hatch of the median chick in that group.

	Estimate	95% Confidence intervals
BA 20		
Early	[2]0.7%[b]	[1]0.3–1.4%
Mid-term	1.4%[ab]	0.8–2.4%
Late	3.4%[a]	1.6–6.9%
BA 30		
Early	5.7%[b]	4.1–7.9%
Mid-term	14.7%[a]	12.0–17.9%
Late	9.3%[ab]	4.8–17.2%
BA 35		
Early	13.7%[a]	10.6–17.6%
Mid-term	14.7%[a]	12.0–17.7%
Late	4.0%[b]	2.0–8.0%
BA 40		
Early	13.6%[a]	10.7–17.2%
Mid-term	14.6%[a]	11.9–17.7%
Late	2.3%[b]	1.0–5.0%

[1] Confidence intervals that are not overlapping within a BA group differ significantly ($P < 0.05$).
[2] Different superscripts within a BA group indicate significant differences ($P < 0.05$).

Table 5
Activity of α-amylase in chicks hatched early, mid-term and late in the hatching window, at hatch and at 6, 10, 20 and 34 days of age. At hatch, α-amylase activity was analysed in samples containing mixed intestine and pancreas. During the growing period, α-amylase activity was analysed in separate intestinal and pancreas samples. Values for hatching groups are averages for the 34 days growth period.

Variable	Hatching group			Age (d)				Pooled SEM	*P*-value		
Mixed pancreas and intestine, α-amylase activity at hatch	Early $n = 5$	Mid-term $n = 5$	Late $n = 5$						Hatching group	–	–
α-Amylase [2]U/g sample	[1]1 874.4	2 834.6	1 577.7	At hatch				253.51	ns	–	–
Intestinal α-amylase activity	Early $n = 20$	Mid-term $n = 20$	Late $n = 20$	6 $n = 15$	10 $n = 15$	20 $n = 15$	34 $n = 15$		Hatching group	Age	Hatching group * age
α-Amylase U/g sample	98.5	120.1	99.3	[3]157.3[a]	114.1[ab]	64.4[b]	88.1[b]	7.93	ns	***	*
Pancreatic α-amylase activity	Early $n = 20$	Mid-term $n = 20$	Late $n = 20$	6 $n = 15$	10 $n = 15$	20 $n = 15$	34 $n = 15$		Hatching group	Age	Hatching group * age
α-Amylase U/g sample	335.0	355.4	398.3	249.4[b]	271.5[b]	501.9[a]	428.9[a]	16.8	ns	***	ns

***$P < 0.001$; *$P < 0.05$; ns $P > 0.05$.
[1] Values are least-squares means (LSM).
[2] U is defined as μmol hydrolysed per minute.
[3] LSM values within rows lacking a common superscript differ ($P < 0.05$).

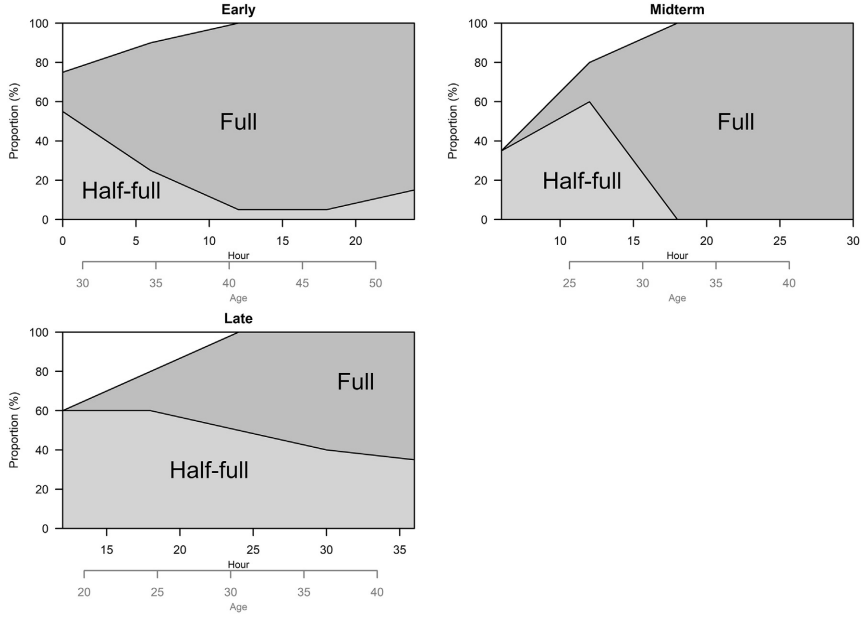

Fig. 1. Level of crop fill in newly hatched chicks. The primary x-axis (black) shows time from examination start and the secondary x-axis (grey) biological age (BA, defined for each hatching group as time (h) since hatch of the median chick). Four individually marked chicks out of 19 chicks per module were examined every 4 h, observed proportions are shown.

Discussion

Many studies have emphasised the importance of immediate access to feed and water in broiler chicks post-hatch (Sklan et al., 2000; Lamot et al., 2014, among others). However, although chicks were offered feed and water from the moment they hatched in this study, 5% eating activity was observed first at a mean BA of 25.4 h. Moreover, it took on average 30.6 h post-hatch before 50% of the birds examined had a full crop. The hatching window in the study was 35 h in accordance with the 37 h long interval reported by Powell et al. (2016), whereas in hatchery practice it would probably have been shorter due to the set pull time. Aviagen, the company that developed the Ross 308 genotype, states that the hatching window for its broiler (time from 1 to 99% hatched chicks) is around 30 h (Tullett, 2009). In the present study, less than 5% of the focal birds in all hatching groups were engaged in eating-related behaviour at the end of the hatching window. In other words, it appears to take some time post-hatch before the chicks are motivated to engage in feed-seeking activities at all.

However, it should be pointed out that, because of the small number of chicks in the present study and the calm environment in the research facility compared with a hatchery, it is possible that chicks hatched in a hatchery would have been stimulated to start eating-related activities earlier. Moreover, the scan sampling methodology takes spot scans, and thus does not cover the birds' activities at all times.

Considering the new knowledge obtained in this study on time to first feed intake, on-farm hatching as a housing system might have greater impacts on production and welfare parameters at farms located far from the hatchery. Despite shorter distances between hatcheries and farms in the Netherlands than in Sweden, benefits for welfare parameters and production performance in on-farm hatched chicks compared with their conventional counterparts have been reported (De Jong et al., 2017). However, these differences may be dependent on factors other than lack of access to feed and water under conventional conditions, such as bacterial load at the hatchery and stress due to climate in the hatchery, handling and transportation. Further studies comparing conventional and on-farm hatching practices should include our findings on time to first feed intake, to obtain reliable decision support for the chicken industry.

Many factors affect the time from the start of incubation to hatch for individual eggs. For example, incubation duration increases with egg size (Wilson, 1991) and the weight of the newly hatched chick has been shown to correlate with the weight of the egg at setting (Tona et al., 2003). This could possibly explain the greater weight of the late-hatched chicks in the present study compared with early and mid-term hatchlings. Age of broiler mother flock and storage time of fertilised eggs also affect the duration of hatch (Sklan et al., 2000).

Table 7
Pair-wise comparisons of crop fill between chicks hatched early, mid-term and late in the hatching window, based on results of an ordinal regression model. Biological age (BA) is defined for each hatching group as time (h) since hatch of the median chick in that group.

	Odds ratio	*P*-value
BA 30		
Mid-term – Early	6.3	0.002
Mid-term – Late	2.5	0.12
Late – Early	2.5	0.16
BA 35		
Mid-term – Early	4.5	0.018
Mid-term – Late	3.6	0.032
Late – Early	1.3	0.73
BA 40		
Mid-term – Early	0.8	0.80
Mid-term – Late	3.0	0.078
Late – Early	0.3	0.094

Biological variation, incubation conditions and hatching synchronisation through species-specific vocalisation also play a part (Tong et al., 2013).

Many studies have concluded that chicks hatched in different parts of a hatching window differ from each other physiologically (Van de Ven et al., 2013; Lamot et al., 2014). Some behavioural differences related to eating have also been observed (Nielsen et al., 2010). Our findings that early hatched chicks were lighter than late-hatched chicks at hatch, but heavier than both late and mid-term hatchings at 3 days of age, correspond well with Lamot et al. (2014) who found that early hatched chicks seemed to have compensated for their low hatching weight by 4 days of age, at which time they had a greater BW than mid-term and late hatchlings. Moreover, Nielsen et al. (2010) observed a minor weight advantage in early hatchlings at 3 days of age. These findings contradict those of Van de Ven et al. (2013), who found no differences due to hatching time with regard to BW or YFBM at hatch. Body weight is a commonly used parameter when assessing chick quality, but BW at hatch may not be a good predictor of post-hatch growth and 1-d BW (i.e. after access to feed and water) has been shown to have higher predictability (Lindholm et al., 2017). Yolk free body mass is also commonly used for assessing chick quality and has the advantage that it corrects for the weight of the residual yolk (Sozcu and Ipek, 2015).

In a study by Dibner et al. (1998), denying chicks access to feed on the day of hatch and the following day resulted in a more pronounced decrease in relative weight of bursa fabricii compared with other organs, an effect that persisted for 21 days. In contrast, early feeding increased bursal weight and also proliferation of B-cells (Dibner et al., 1998). In the present study, the early hatching group had lower relative bursal weight than the mid-term hatching group at hatch. Even if it took longer for the early hatchlings to start to eat compared with the mid-term and late groups, the birds chosen for post-hatch dissection were still euthanised before they had the chance to eat or drink. Therefore the explanation for the difference in organ weight presented by Dibner et al. (1998) is not applicable here.

Relative intestinal weight was greater in the mid-term group than in the early and late groups at hatch. The late group also had greater relative intestine weight than the early group when considering the whole experimental period. The greater relative length of intestine in the early and mid-term groups compared with the late group at hatch is also worth noting. Intestinal growth by elongation has been shown to be regulated by contraction of the smooth muscle cells already in the embryonic state. As embryogenesis progresses, differentiation of these smooth muscle cells depresses elongation (Khalipina et al., 2019). Varying effectiveness of this process during embryogenesis might be responsible for the differences in intestinal length at hatch, and needs further investigation.

No effect of hatching group on intestinal or pancreatic α-amylase activity was observed either at hatch or later during the study. In a study comparing poults 24 h post hatch, decreased activity of pancreatic α-amylase were observed in poults supplemented with a liquid nutrient mix composing glucose, starch and oil compared with poults kept feed-restricted post-hatch (Pinchasov, 1994). This indicates that the presence of feed in the intestine may not be of crucial importance for the onset of enzymatic activity (Pinchasov, 1994). It could well be the reason why differences in onset of feed intake and foraging observed between hatching groups in the present study did not result in any differences in α-amylase activity in the intestine or pancreas. On the other hand, Gracia et al. (2003) highlight that the early development of the gastrointestinal tract is stimulated by feed intake and also the importance of early synthesis of pancreatic enzymes to counteract negative effects on growth post-hatch. Moreover, Svihus (2014) states in a review that broiler chicks, when fed early post-hatch, have a high amylase activity.

Pancreatic α-amylase activity increased with age, confirming results by Noy and Sklan (1995). Intestinal α-amylase activity decreased with age, in contrast with earlier findings (Nir et al., 1993; Noy and Sklan, 1995). Why the age-dependent increase in pancreatic α-amylase did not bring about a corresponding increase in intestinal α-amylase activity in our study is not known. However, Nitsan et al. (1991) observed a decrease in intestinal α-amylase from 9 to 15 days of age, which is more in line with our results. Moreover, in a study comparing fast-growing broilers and slow-growing layer cockerels, Zelenka and Čerešňáková (2005) found that overall starch digestibility was linearly decreasing with age in the broilers.

In conclusion, the time of hatch affected some of the parameters studied. The observed differences in organ weights at hatch did however not persist throughout the production cycle. Neither did the differences in organ weights reflect themselves in the BW differences, because the BW differences were no longer apparent at 10 days of age. Even though there were some differences in early eating behaviour and crop fill in early life, the chicks seemed capable of compensating for these during the grow-out period.

Ethics approval

The experimental set-up was approved by Uppsala Animal Experiment Ethics Board (application reference number C 36/16).

Data and model availability statement

The statistical software SAS 9.4 and R version 3.4.0 were used for analysis of data. None of the data were deposited in an official repository. Models are available upon request.

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Declaration of interest

The authors have no conflict of interest to declare.

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II

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Effects of access to feed, water, and a competitive exclusion product in the hatcher on some immune traits and gut development in broiler chickens

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ABSTRACT

1. This study evaluated the effect of access to feed, water, and the competitive exclusion (CE) product Broilact®, administered in the hatcher, on broiler performance, caecal microbiota development, organ development, intestinal morphology, serum levels of IgY and vaccine-induced antibody responses.
2. In total, 250 chicks were hatched in a HatchCare™ hatcher and divided into four groups, given access to feed, water and the CE product sprayed on the chicks (CEs); access to feed, water, and the CE product in water (CEw); access to feed and water (Cpos); or no access to feed and water (Cneg) in the hatcher.
3. At the research facility, 10 chicks per hatching treatment were euthanised for organ measurements. The remaining 200 chicks were randomly distributed to 20 pens. On d 11, all birds were vaccinated against avian pneumovirus (APV). Three focal birds per pen were blood-sampled weekly for quantification of IgY and serum antibodies to APV. On d 11 and 32, two birds per replicate pen were euthanised for organ measurements and sample collection. Feed intake and body weight were recorded weekly.
4. Delayed access to feed and water reduced weight gain and feed intake early in life. At the end of the study, no differences in body weight remained.
5. There were some early effects on organs, with depressed intestinal development and higher relative gizzard weight for the Cneg group at placement. No treatment effects on the immune traits measured were detected.
6. The relative abundance of seven bacterial genera differed between treatment groups at d 11 of age. The results suggested that chickens are capable of compensating for 40 h feed and water deprival post-hatch. Provision of Broilact® did not have any persistent performance-enhancing properties, although different outcomes under rearing conditions closer to commercial production cannot be ruled out.

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Introduction

The majority of broiler chickens intended for meat production start their lives at a commercial hatchery. There, brooded eggs are inserted into the hatcher and, depending on factors such as egg storage time prior to brooding, broiler breeder age, and biological variation, each batch of eggs will hatch over a period of 24–48 h. This period is referred to as the 'hatching window' and may generate problems, if it is too long. In conventional practice, chicks are given their first access to feed and water at the rearing farm. After loading and transportation, particularly early-hatched chicks may be feed-deprived for up to 72 h post-hatch on arriving at the rearing farm (Willemsen et al. 2010).

Chicks that have been deprived of feed and water post-hatch have been shown to have lower utilisation rate of the yolk sac, which may have a negative impact on the uptake of maternal antibodies transferred from the mother hen to the chick *via* this temporary organ (Gonzales et al. 2003). The chick's immune system at hatch is still immature and the chick is therefore dependent on these maternal antibodies to withstand pathogens in the surrounding environment. Moreover, at hatch the gut is susceptible to bacteria, whether pathogenic or favourable (Lan et al. 2005). The early responsiveness of the gut makes it possible to colonise it artificially with bacteria that have been shown to be beneficial for chick gut health (Seifi et al. 2017). Favourable bacteria that are deliberately added to the diet are called probiotics and are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO 2002). Probiotic bacteria can act beneficially through several different activities. One of these is competitive exclusion, whereby the bacteria bind to receptor sites in the gut epithelium (Seifi et al. 2017), blocking harmful bacteria from colonising these sites and impacting the host. Supplementation with probiotics is well known to have immuno-modulatory effects and has been shown for example to increase the relative weight of the spleen and bursa, organs important for the immune response (Karimi Torshizi et al. 2010). Supplementation with *Lactobacilli* spp. has been shown to increase serum levels of immunoglobulin (Ig)Y and IgM in broiler chickens (Koenen et al. 2004). In Finland, most broiler chickens are given the competitive exclusion (CE) product Broilact® (Orion Corporation, Espoo, Finland), consisting of normal microflora of poultry derived from the caecum of healthy hens (Schneitz and Hakkinen

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2016). The main constituent bacterial groups of the product (at genus level) have been determined as *Escherichia* (named *Escherichia-Shigella*, 42.2–43.14%), *Enterococcus* (14.06–17.18%), *Bacteroides* (11.04–12.57%), and *Lactobacillus* (6.6–8.62%) spp. in a previous study (Such et al. 2021). For chicks hatched in hatcheries, the product may mimic the natural transfer of a healthy microbiota from mother hen to chick. Broilact® is provided in the drinking water or as an aerosol sprayed on the down of the chicks.

The aim of this study was to investigate whether adapted management routines immediately post-hatch can be beneficial for chicken immune response and growth. The effects of access to feed and water already in the hatcher, in combination with Broilact® supplied in the drinking water or as an aerosol of water sprayed on the down of the chicks, were studied. Variables of interest included growth, feed intake, serum levels of IgY, vaccine-induced antibody responses, intestinal development, and gut microbiota.

Materials and methods

Procedure at the hatchery

All chicks included in the study were hatched in a HatchCare™ hatcher manufactured by HatchTech. The baskets in a HatchCare™ hatcher have cavities into which feed is distributed prior to emergence. By stretching their heads out of the box, chicks can reach water in gutters lining the wall of the hatcher. The HatchCare™ system provides an illuminated environment for hatching in bright light. At the participating hatchery (located in southern Sweden), a total of 250 chicks with wet down were collected from the boxes during a period of 3 h, in order to reduce the variation in the hatching window. These chicks were randomly distributed to one of four treatments. Chicks were divided into baskets according to group where each treatment had their own separate water through. Treatment groups were: i) a negative control group that received neither feed nor water (Cneg); ii) a positive control group that received feed and water during hatch (Cpos); iii) a Broilact® in water group (CEw) that had access to feed and water with Broilact® added; and iv) a Broilact® spray group (CEs) that had access to feed from the beginning and received water when the droplets sprayed on the down had been consumed and/or dried. Chicks in the groups that were provided feed (Cpos, CEw and CEs) were given a commercial pre-starter feed including a coccidiostat (Lantmännen, Falkenberg) at the hatchery.

When all birds had been collected and placed in the hatcher according to the treatment schedule, fresh water with Broilact® added was provided in the water trough of the CEw group every 4 h for a total of 12 h. Before adding the fresh Broilact® solution, the residual solution in the water trough was drawn out using a syringe and consumption was calculated. Mean total consumption of Broilact® per bird was approximately 2 mg during the 12 h of supplementation. When the birds had started to drink in the boxes with immediate access to water, the 60 chicks in the CEs group were evenly sprayed with Broilact® solution (1 mg of Broilact® per 0.3 ml regeneration agent water solution per chick) at approximately 12 h after cease of placement, according to the dose recommendation protocol provided by Orion Corporation, using a handheld spray bottle.

Transportation, placement, and feed

After pull, sorting and standard hatchery quality control (approximately 1 h), the birds were transported (approximate transportation time 16 h) to the Swedish Livestock Research Centre at the Swedish University of Agricultural Sciences, Uppsala, where they were given access to feed and water 17 h after pull. The chickens in the study were hatched approximately 24–27 h prior to pull, meaning that chicks in the Cneg group had been without access to feed and water for approximately 40 h on arriving at the research facility. The remaining treatment groups were given continued access to regular water pending transportation but they had no access to feed after sorting and were thus without access to water and feed for about 15 and 16 h, respectively, post-pull. Immediately on arrival at the research facility, 10 birds per treatment group ($n = 40$ in total) were euthanised for dissection and organ excision and weighing. The remaining chicks from each hatchery treatment were randomly distributed to five replicate modules with 10 chicks in each, resulting in 200 chicks distributed over 20 modules. Three focal birds per replicate (module) were wing-tagged so that blood samples could be taken from the same birds throughout the study. Each module measured 1.5 m × 0.75 m and contained a feeder and three nipple drinkers and was bedded with wood shavings. On arrival, the temperature in the research facility was set to 33°C. After 3 d, the temperature was successively lowered to reach 23°C at 24 d and remained so for the rest of the study. Constant light was provided on the day of arrival and the following day. On the third day, the chicks were given 1 h of darkness between 11 pm and midnight. Thereafter, the chicks were provided with 1 h of extra darkness per night until d 8. From this point until the end of the study, the lights were off between 11 pm and 5 am. Day 0 was defined as the time when most chicks hatched, *i.e.*, during embryonic d 20 (ED20). From day of placement (d 2) until d 10, the chicks were provided with a commercial starter feed. On d 11, the starter feed was replaced with a commercial grower feed (both feeds Svenska Foder AB, Lidköping, Sweden). No coccidiostats were included in the feed given at the research facility. Feed samples were analysed for dry matter, crude protein, crude fibre, ash, and fat (as ether extract). Sub-samples were dried for 16 h at 103°C for analysis of dry matter (DM). Ash was analysed according to Jennische and Larsson (1990), after incineration for 3 h at 550°C. The European Community (1998) methodology was used for analysis of ether extract, while crude protein content (Nx6.25) was measured according to the Kjeldahl method (Nordic Committee on Food Analysis 2003). Analysed chemical composition of the starter feed was ash 74 g/kg DM, crude protein 241 g/kg DM, crude fibre 41 g/kg DM, and ether extract 69 g/kg DM, and that of the grower feed was ash 52 g/kg DM, crude protein 237 g/kg DM, crude fibre 40 g/kg DM, and ether extract 63 g/kg DM. The calculated metabolisable energy content was 12.8 ME MJ/kg DM for the starter feed and 13.1 ME MJ/kg DM for the grower feed.

Growth, feed intake and organ development

Organ weight (yolk sac, small intestine with content, spleen, bursa, heart, liver, proventriculus, gizzard with contents and rinsed gizzard) and length (body length, small intestine with contents) were recorded from the 10 euthanised birds per

treatment (two birds per replicate) at 2 (prior to placement at the research facility), 11 and 32 d of age. Birds at 2 and 11 d of age were euthanised by neck dislocation following stunning with a blow to the head. Birds at 32 d of age were euthanised by a 100 mg/ml intravenous injection of pentobarbital sodium in the wing vein. Chicken weight and feed consumption per module were recorded weekly. Mortality was recorded daily.

Histology: villi height, villi width, and crypt depth in duodenum

From the chicks sacrificed for organ measurements at two and 11 d of age, the small intestine was rapidly removed and a 3-cm-long piece of duodenum, distal to the duodenal loop, was excised. The tissue was cut open, pinned to a small rectangle of cork to minimise distortion and fixed in glutaraldehyde (2.5%, pH 7.2) overnight. It was then rinsed in phosphate buffer (1/15 M, 7.2 pH) and trimmed into 2 mm thick transverse slices, which were dehydrated in increasing concentrations of ethanol and embedded in water-soluble resin (Leica Historesin, Heidelberg, Germany). Sections (2 µm) of resin-embedded duodenum were stained with haematoxylin-eosin for evaluation by light microscopy. Before evaluation, all slides were coded, to avoid bias due to the observer, and digital images of duodenum sections were taken with a Nikon Microphot-FXA microscope using a 4× objective lens (Bergström Instrument AB, Stockholm, Sweden). Five consecutive villi per sample were measured. The villi chosen had to have an intact lamina propria and a single epithelial cell layer, to avoid including samples that could have been cut askew. Only representative villi that were judged not to have been affected by preparation and that were free from artefacts were chosen. Villi where the tip ends were diffuse or those with invisible crypts were not selected for analysis. Crypts were measured in the same direction as the villi base, from the branching to the start of the muscularis mucosa. Villi width was measured beneath the villi tip where the epithelial cell nuclei had straightened out and were no longer at an angle to the tip. Villi width was measured perpendicular to the tip (Figure 1).

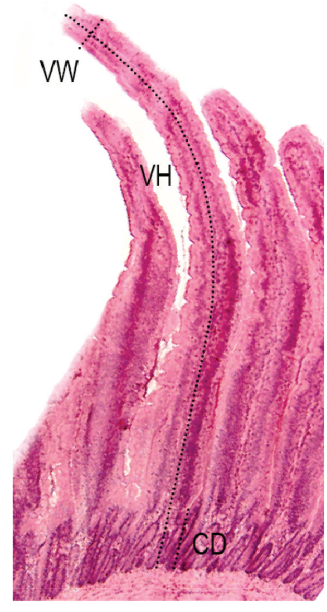

Figure 1. Histological image of the duodenum. Villi height (VH) was measured from the tip of the villus to the start of the muscularis mucosa. Crypt depth (CD) was measured in the same direction as the villi base, from the branching to the start of the muscularis mucosa. Villi width (VW) was measured beneath the villi tip where the epithelial cell nuclei had straightened out and were no longer at an angle to the tip. Villi width was measured perpendicular to the tip.

Quantification of serum levels of IgY and vaccine-induced antibody responses

Blood samples from 15 focal birds per treatment were collected from the jugular vein into test tubes without additive at 3, 11, 18, 25 and 31 d of age. These samples were stored for 24 h at room temperature before centrifuging for 10 min at 10 000 × g. Serum was collected and stored at −20°C prior to analysis with ELISA methodology.

The total amount of IgY in serum from all sampling occasions was analysed using the Chicken IgG ELISA Quantitation Set (Cat. No. E30–10) manufactured by Bethyl Laboratories Inc. (U.S.A) and the ELISA assay was set up according to the manufacturer's protocol. The assay was performed in flat-bottomed 96-well plates (MaxiSorp, Nunc™, ThermoFisher Scientific, www.thermofisher.com). An in-house substrate buffer (1 mM 3,5,3',5'-tetrametylbenzidine in 0.1 M potassium citrate, pH 4.2, with 0.007% H_2O_2) was used for visualisation of antibody binding. The colour reaction was stopped with 2 M H_2SO_4 at a standardised time point and the A_{450} value was measured in an ELISA reader. The total IgY concentration in the samples was calculated by linear regression from serial dilutions of the chicken IgY standard included in the kit. The linear range of detection of the ELISA assay was between 25 and 200 ng IgY/ml.

After blood sampling on d 11, all birds were vaccinated with commercial vaccine Nobilis RT Inac vet (MSD Animal Health) against avian pneumovirus (APV). All birds were injected intramuscularly with 0.5 ml vaccine into the breast muscle. Serum samples from d 11 and 31 were analysed for antibodies to APV, using the Avian Pneumovirus Antibody Test Kit (06-44 300-04) manufactured by IDEXX Laboratories Inc. (U.S.A) according to the manufacturer's protocol. Samples were tested in duplicate and, to increase the detection limit, serum was diluted 1:100, rather than the recommended 1:500. Results were expressed as absorbance values at 650 nm and a cut-off value for samples deemed positive for antibodies to APV was calculated as the mean absorbance value +2 standard deviations for all pre-vaccination samples collected at d 11 ($n = 117$).

Gut microbiota

At d 2, the contents from both caeca were collected from 10 birds per treatment. On d 11 and 32, samples were collected from two birds per replicate euthanised for organ sampling. In total, 120 samples were collected with an aseptic procedure, immediately frozen in liquid nitrogen, and thereafter stored at −80°C until extraction.

DNA extraction and sequencing

DNA was extracted from 180 to 220 mg caecal contents from 120 samples in total (four treatments, three ages, and 10 replicates per treatment at each age) using a QIAamp Fast DNA Stool Mini Kit (CatNo. 51604, Qiagen, Germany). Due to technical reasons, one sample from Cneg at d 2 and one sample from CEs at d 32 were missing in the analysis. The kit was used according to the manufacturer's instructions with some minor changes, including use of bead beating to break down bacterial cell walls. In brief, 0.3 g sterilised 0.1 mm zirconia/silica beads (BioSpec Products, Bartlesville, U.S.A) and 1 ml InhibitEX Buffer from the QIAamp Fast DNA Stool Mini Kit were added to each sample and homogenised by vortexing for 1 min. The suspension was heated for 5 min at 70°C to lyse cells. Samples were then cooled on ice before running in the Precellys24 sample homogeniser (Bertin Technologies, Montigny-le-Bretonneux, France) at 1 × 60 s at 60 × *g* for two rounds, with 5 min on ice in between. Samples were then centrifuged for 5 min at 9600 × g to pellet particles. The supernatant (700 µl) was pipetted to new 1.5 ml tubes and centrifuged again for 5 minutes at 17 000 × *g*. Thereafter 400 µl of the supernatant were mixed with 30 µl proteinase K and 400 µl AL buffer and vortexed for 15 s, followed by incubation at 70°C for 10 min. A further 400 µl 99.5% ethanol were then added before vortexing again. Lysate was added (2 × 600 µl) to clean QIAamp spin columns and centrifuged at full speed (21 100 × *g*) for 3 min. Each QIAamp spin column was placed in a new collection tube, AWI buffer (500 µl) was added, and the tube was centrifuged again for 1 min at full speed. The column was then moved to a new collection tube and AW2 buffer (500 µl) was added, followed by centrifuging for 3 min at full speed. The columns were placed in clean collection tubes and centrifuged empty for 1 min before being moved to Eppendorf tubes. The DNA was eluted with 100 µl buffer and stored at −20°C for delivery to Novogene (Beijing, China). The library of 16S rRNA gene was constructed and sequenced at Novogene using the Illumina HiSeq 2500 platform. In brief, the V3-V4 region of 16S rRNA gene was amplified with primers 341F (CCTAYGGGRBGCASCAG) and 806 R (GGACTACNN GGGTATCTAAT). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs).

Bioinformatic analysis

The raw sequencing data have been deposited in database of the National Centre for Biotechnology Information (NCBI), under accession number PRJNA813981. Bioinformatic data processing was performed using Quantitative Insights into Microbial Ecology 2 (Core 2019.04; Bolyen et al. 2019). The barcode and primer sequence of raw demultiplexed reads were trimmed off. The trimmed reads were further processed using DADA2 to denoise, dereplicate reads, merge pair end reads, and remove chimeras (Callahan et al. 2016), using truncation length of 221 bp for both forward and reverse reads. A phylogenetic tree was built using FastTree and MAFFT alignment (Katoh et al. 2002; Price et al. 2010). The SILVA SSU Ref NR 99 132 dataset was first trimmed to the corresponding primer region and trained as classify-sklearn taxonomy classifier (Pedregosa et al. 2011; Quast et al. 2013; Bokulich et al. 2018). The amplicon sequence variants (ASV) were then assigned taxonomy using the resulting classifier. After trimming and quality filtering, the sequencing of 16S rRNA gene yielded a total of 7,793,838 sequences from 118 samples. A minimum of 27 311 sequences per sample was used for rarefying the number of reads per sample (Weiss et al. 2017). The generalised UniFrac distance matrix (alpha = 0.5) and alpha rarefaction were generated using the QIIME2 diversity plugin (Chen et al. 2012; Bolyen et al. 2019).

Statistical analyses

Analysis of data on growth, feed conversion ratio (FCR), feed intake, organ weight, and histology was performed using the statistical program SAS (version 9.4). All data were analysed using the procedure mixed (PROC MIXED) statement, with hatching treatment as fixed factor and module as random factor. At d 2, module was not included in the random statement for organ and histology data, because the chicks were yet to be assigned to modules. Organ weights (d 11 and 32) were analysed with age as an additional fixed factor and a repeated statement. The unstructured UN covariance structure was primarily used and replaced with the first order autoregressive AR (1) when needed. Antibody data were presented as mean values with 95% confidence intervals. Mean values with non-overlapping confidence interval were treated as rejecting the null hypothesis of no difference. Fisher's exact test was used to investigate whether there were significant differences in proportions of positive and negative responders to APV between groups. Microbial differences due to hatching treatment and age at the phylum, class, order, family, genus and ASV level were analysed with ANCOM methodology (Mandal et al. 2015). To investigate the microbial difference between hatching treatment at genus level on d 11, the rarefied ASV table was used to select the genera that had a relative abundance (RA) higher than 1%. The selected genera were analysed with quasi-Poisson generalised linear models using R (https://r-project.org).

Results

Body weight, length and organ development at placement

On arrival at the research facility on d 2, the organ data collected from 10 chicks per hatching treatment group revealed no differences in body weight, chick length, or yolk sac weight (in g or as proportion of body weight; Table 1). There was a tendency for a difference in yolk-free body mass (YFBM), *i.e.*, body weight excluding yolk weight, with lower weight in the Cneg group compared with all other groups. There were no differences in the relative weight of spleen, bursa, heart, liver, or of proventriculus and gizzard when weighed together. However, there was a difference in intestinal weight (expressed as a proportion of total body weight), with the CEw, CEs, and Cpos groups having heavier intestines than the Cneg group. The CEw group had greater relative intestine weight than the Cpos group. In addition, there was a difference in absolute numerical terms (data not shown), with the Cneg group having lighter intestines ($P < 0.0001$; 2.5 g) than the Cpos (3.39 g), CEw (3.71 g) and CEs (3.45 g) groups. Regarding intestine length, the Cneg group had shorter intestines than all other groups. The Cneg group also had shorter intestines in numerical terms (48.1 cm, $P < 0.0001$) than Cpos (57.95 cm), CEw (57.4 cm), and CEs (58.25 cm). Moreover,

Table 1. Body, yolk sac, and organ weight at 2 d of age in chicks subjected to four different treatments in the hatcher: no access to feed and water (Cneg), access to feed and water (Cpos), access to feed, water, and a competitive exclusion product (CE) provided in the water (CEw), or access to feed, water, and a CE product sprayed on the down of the newly hatched chicks (CEs). Values shown are based on measurements on individual birds.

	Hatching treatment					P-value
Variable	Cneg n=10	Cpos n=10	CEw n=10	CEs n=10	SEM	Hatching treatment
Body weight (g)	[1]41.5	44.1	44.3	44.0	0.98	0.1588
Chick length (cm)	21.2	20.4	20.8	20.9	0.36	0.4272
YFBM (g)	39.8	42.4	42.9	42.6	0.94	0.0925
Yolk sac (g)	1.69	1.76	1.35	1.43	0.18	0.2999
Yolk sac (g/kg bw)	40.6	39.4	30.7	32.5	3.86	0.1947
Small intestine (g/kg bw)	[a]60.3[c]	76.8[b]	83.8[a]	78.4[ab]	1.88	<.0001
Small intestine (cm/kg bw)	1161[b]	1317[a]	1298[a]	1327[a]	36.6	0.0091
Spleen (g/kg bw)	0.30	0.37	0.47	0.38	0.047	0.1350
Bursa (g/kg bw)	1.67	1.55	1.43	1.51	0.115	0.5312
Heart (g/kg bw)	8.91	9.30	9.20	9.19	0.327	0.8480
Liver (g/kg bw)	30.0	30.0	30.4	31.7	0.682	0.2902
Proventriculus & gizzard (g/kg bw)	73.3	81.6	77.9	76.7	2.58	0.1770
Gizzard full (g/kg bw)	61.3	69.7	66.4	65.0	2.22	0.0895
Gizzard empty (g/kg bw)	59.1[a]	53.9[b]	53.0[b]	53.7[b]	1.2	0.0039

[1]Values are least squares means (LSM).
[2]LSM values within rows lacking a common superscript are significantly different ($P<0.05$).
***$P<0.001$; **$P<0.01$; *$P<0.05$; ns $P>0.05$.

the Cneg group had the heaviest gizzard, when weighed emptied and rinsed, of all the groups (Table 1).

There were no differences between treatments in absolute numerical gizzard weight when weighed emptied and rinsed ($P < 0.7678$; Cneg: 2.46 g; Cpos: 2.38 g; CEw: 2.35 g, CEs: 2.37 g). However, there was a tendency ($P = 0.0895$) for a difference in full relative gizzard weight, where Cpos had the numerically highest and Cneg the numerically lowest weight.

Growth, FCR, feed intake, and organ development during the growing phase

The Cneg group had lower body weight than all other groups from 2 to 11 d (Table 2). At 18 d, Cneg still had lower body weight compared with CEs and Cpos. Moreover, CEw had lower body weight compared with the CEs and Cpos groups. At 25 d, the difference in body weight persisted only between Cneg and Cpos. At 32 d of age, there were only slight differences between treatments, and these were no longer significant after adjustment using the Tukey's test.

At 4 d of age, the Cneg group had lower feed intake (FI) than the CEw group. At 11 and 18 d of age, Cneg had lower FI than all other groups, while at 25 and 32 d of age Cneg had lower FI than the Cpos and CEs groups.

There were some differences in FCR during the grow-out period. At 18 d of age, the CEw group had inferior FCR to the Cpos group, while at 25 and 32 d of age the CEw group had poorer FCR compared to the other groups (Table 2).

No effects of hatching treatment on organ weight and length were observed during the grow-out period (Table 3). However, there was an effect of age, with YFBM (g) and relative weight of spleen and bursa increasing with age. Moreover, a decrease in proportional weight or length was

Table 2. Body weight, accumulated feed intake (FI), and feed conversion ratio (FCR) at six different ages in chickens subjected to four different treatments in the hatcher: no access to feed and water (Cneg), access to feed and water (Cpos), access to feed, water, and a competitive exclusion (CE) product provided in the water (CEw), or access to feed, water and a CE product sprayed on the down of the newly hatched chicks (CEs). Values are hatching group mean values.

	Hatching Treatment					
	Cneg n = 5	Cpos n = 5	CE_W n = 5	CE_S n = 5	SEM	*P-value*
Body weight (g)						
2	[1]40.7[b]	45.7[a]	45.2[a]	[2]45.2[a]	0.66	<.0001
4	70.9[b]	85.1[a]	81.7[a]	82.6[a]	1.07	<.0001
11	278[b]	322[a]	303[a]	312[a]	4.77	<.0001
18	688[b]	782[a]	704[b]	769[a]	15.4	<.0011
25	1283[b]	1428[a]	1313[ab]	1399[ab]	29.3	0.0095
32	2027	2195	2034	2180	48.0	0.0396[3]
FI (G)						
4	26.5[b]	34.9[ab]	36.7[a]	35.1[ab]	2.88	0.0191
11	273[b]	311[a]	302[a]	306[a]	6.24	0.0003
18	791[b]	886[a]	841[a]	876[a]	14.0	0.0010
25	1621[b]	1797[a]	1706[ab]	1766[a]	28.6	0.0030
32	2748[b]	2984[a]	2854[ab]	2965[a]	49.5	0.0145
FCR						
4	0.92	0.89	1.02	0.95	0.082	0.5258
11	1.14	1.12	1.16	1.14	0.019	0.1904
18	1.21[ab]	1.20[b]	1.26[a]	1.21[ab]	0.013	0.0166
25	1.31[b]	1.31[b]	1.37[a]	1.32[b]	0.015	0.0028
32	1.37[b]	1.38[b]	1.44[a]	1.39[b]	0.012	0.0083

[1]Values are least squares means (LSM).
[2]Values within rows lacking a common superscript are significantly different ($P < 0.05$).
[3]No statistically significant differences between treatments could be demonstrated using Tukey's test.
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns $P > 0.05$.

Table 3. Body and organ weight at 11 and 32 d of age in chickens subjected to four different treatments in the hatcher: no access to feed and water (Cneg), access to feed and water (Cpos), access to feed, water, and a competitive exclusion (CE) product provided in the water (CEw), or access to feed, water and a CE product sprayed on the down of the newly hatched chicks (CEs). Values are based on measurements on individual birds.

	Hatching group					Age			P-value		
Variable	Cneg n = 5	Cpos n = 5	CE_W n = 5	CE_S n = 5	SEM	11 n = 20	32 n = 20	SEM	Hatching treatment	Age	Hatching treatment *age
Body weight (g)	[1]1153	1235	1220	1237	25.7	[2]18[b]	2105[a]	14.5	0.1100	<.0001	0.2038
YFBM (g)	1153	1235	1220	1236	25.8	317[b]	2104[a]	14.5	0.1125	<.0001	0.1994
Intestine (g/kg bw)	70.0	70.1	71.9	72.0	0.97	85.4[a]	56.6[b]	0.83	0.3030	<.0001	0.7146
Intestine (cm/kg bw)	225.2	212.7	218.8	221.8	6.04	353.2[a]	86.0[b]	3.35	0.5282	<.0001	0.8263
Spleen (g/kg bw)	0.86	0.86	0.90	0.79	0.054	0.71[b]	1.00[a]	0.036	0.5699	<.0001	0.1046
Bursa (g/kg bw)	2.00	1.87	1.98	1.88	0.112	1.79[b]	2.08[a]	0.070	0.7860	0.0036	0.1452
Heart (g/kg bw)	7.79	7.20	7.33	7.20	0.202	8.38[a]	6.38[b]	0.160	0.1682	<.0001	0.8272
Liver (g/kg bw)	30.9	33.5	31.4	31.7	1.157	36.8[a]	26.9[b]	0.736	0.4324	<.0001	0.7171
Proventriculus & gizzard (g/kg bw)	36.7	35.6	35.3	34.5	0.724	45.6[a]	25.4[b]	0.583	0.2094	<.0001	0.6443
Gizzard full (g/kg bw)	30.7	29.9	29.5	28.9	0.655	38.4[a]	21.2[b]	0.539	0.3086	<.0001	0.4043
Gizzard empty (g/kg bw)	19.8	18.7	18.6	18.8	0.395	24.8[a]	13.2[b]	0.305	0.1496	<.0001	0.3230

[1]Values are least squares means (LSM).
[2]LSM values within rows lacking a common superscript are significantly different ($P < 0.05$).
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns $P > 0.05$.

observed when considering intestinal weight, intestinal length, heart, liver, proventriculus, and gizzard weighed together, as well as gizzard alone, either with contents or emptied and rinsed (Table 3).

Intestinal development

There were no differences between hatching treatments with regard to villi height, width, crypt depth or the ratio between villi height and crypt depth at 2 or 11 d of age (Table 4). There was a tendency ($P = 0.0654$) for a difference in crypt depth at 2 d of age, with Cpos having numerically more shallow crypts. A corresponding tendency ($P = 0.0978$) in the ratio between villi height and crypt depth was recorded at the same age.

Total levels of IgY in serum and vaccine-induced antibody responses

Total concentration of IgY in serum was monitored throughout the experiment (Figure 2). The results showed that in general, all chicks had the highest observed levels of IgY in serum on d 3. The serum levels of maternally derived antibodies then rapidly declined and serum IgY showed the lowest observed levels on d 18 (approximately 10% of d 3 levels). Thereafter, serum IgY levels were found to be slightly increased on d 25 and 31 (to approximately 20% of d 3 levels). To reduce the influence of variation between individuals, individual IgY levels relative to d 3 values were also calculated (data not shown). However, no differences in total IgY levels, either as actual or relative amounts, were observed between treatments during the experiment.

All chicks were vaccinated with an inactivated APV vaccine at 11 d of age and specific antibody levels to APV were recorded on d 11, prior to vaccination, and on d 31, 20 d after vaccination (Figure 3). Based on pre-vaccination serum values, a technical cut-off value for detection of antibodies to APV was calculated as Abs_{650} 0.086. Based on this definition, 44% of the chicks responded with antibody production after vaccination, although substantial antibody responses were observed for fewer individuals (Figure 3). No difference in APV antibody levels or in the proportion of responding individuals was observed between the treatment groups (CEs 47%, CEw 36%, Cpos 33%, Cneg 60%). Overall, chickens that were deemed positive for vaccine-induced antibody production also showed higher total serum IgY levels (1.131 ± 0.174

Table 4. Villi height and length and crypt depth in intestinal sections sampled at 2 and 11 d of age in chicks given four different treatments in the hatcher: no access to feed and water (Cneg), access to feed and water (Cpos), access to feed, water, and a competitive exclusion (CE) product provided in the water (CEw), or access to feed, water, and a CE product sprayed on the down of the newly hatched chicks (CEs). Values are based on measurements on individual birds.

	Hatching treatment					P-value
Variable (µm)	Cneg n = 5	Cpos n = 5	CE_W n = 5	CE_S n = 5	SEM	Hatching group
Villi height						
2d	[1]699.5	585.5	669.6	704.6	48.0	0.3034
11d	1138	1160	1276	1265	81.2	0.5327
Villi width						
2d	92.6	89.1	97.1	100.5	5.63	0.5152
11d	116.5	134.2	124.5	134.5	10.8	0.6046
Crypt depth						
2d	93.5	61.7	85.7	92.2	8.64	0.0654
11d	126.9	148.1	154.9	134.2	9.49	0.1864
Ratio[2]						
2 d	7.51	9.86	8.14	7.81	0.666	0.0978
11 d	9.12	7.85	8.23	9.67	0.628	0.1991

[1]Values are least squares means (LSM).
[2]Ratio is defined as villi height divided by crypt depth.

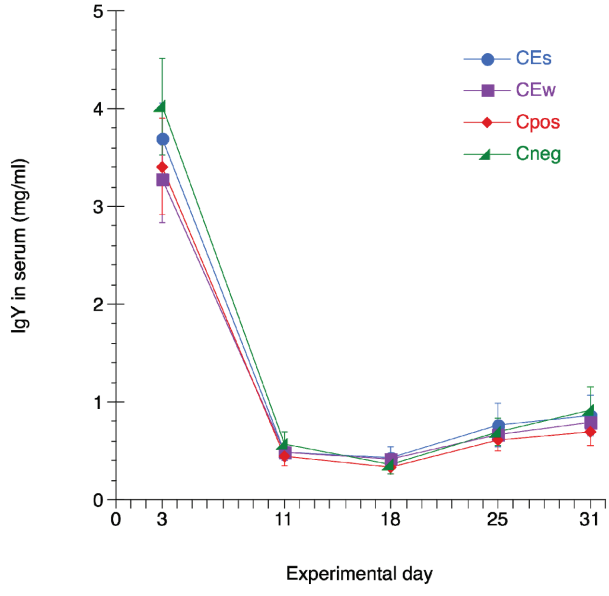

Figure 2. Total amounts of IgY in serum collected from chickens at 3, 11, 18, 25 and 31 d of age. Values are group mean ±95% confidence interval. Treatments: chicks in the hatcher were given access to feed, water, and a competitive exclusion (CE) product sprayed on the down of the newly hatched chicks (CEs; circles), access to feed, water, and a CE product provided in the water (CEw; squares), access to feed and water only (Cpos; diamonds), or no access to feed and water (Cneg; triangles).

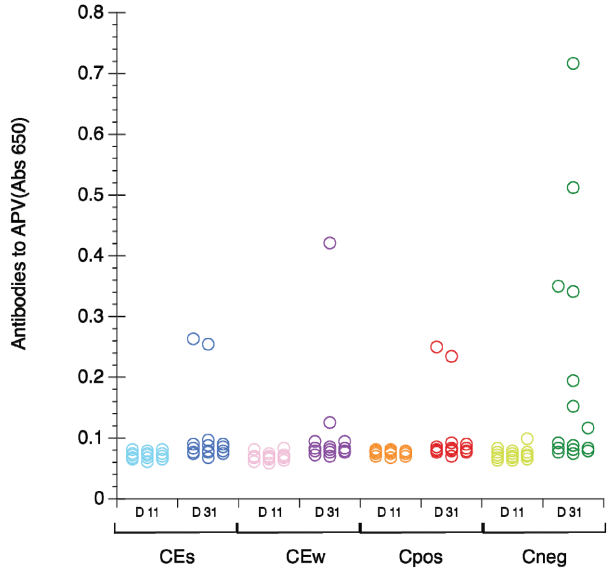

Figure 3. Antibodies to avian pneumovirus (APV) in serum samples collected before vaccination against APV at d 11 and 20 after vaccination at d 31. Results shown are absorbance 650 nm values for individual chickens in the four treatment groups. The cut-off value for samples testing positive for antibodies to APV was calculated to be 0.086 (for details, see Materials and Methods). Treatments: chicks in the hatcher were given access to feed, water, and a competitive exclusion (CE) product sprayed on the down of the newly hatched chicks (CEs), access to feed, water, and a CE product provided in the water (CEw), access to feed and water only (Cpos), or no access to feed and water (Cneg).

mg IgY/mL serum) at d 31 compared with negative chickens (0.700 ± .083 mg IgY/mL serum) (mean ±95% confidence interval; $n = 57$ and $n = 59$, respectively).

Microbial populations

The 16s rRNA gene sequences were distributed in 807 amplicon sequence variants (ASV), representing 91 taxonomic families and 179 genera. The rarefaction curves of observed ASV revealed an effect of age (Figure 4(a)). As the age of chicks increased, the average number of observed ASV increased from 53 at d 2 to 187 at d 11 and 258 at d 32. A principal coordinate analysis (Poi) plot of generalised UniFrac distance matrix revealed an effect of age, whereas treatments did not show clear effects (Figure 4(b)). Four samples from the CEw group and one sample from CEs at d 11 were clustered closer to d 32, and one sample from CEw at d 32 was closer to d 11. The relative abundance of *Bacteroides* and *Alistipes* most likely explained this clustering pattern, with higher levels of these two genera associated with samples at d 32.

An effect of treatment on the microbial composition at genus level was observed at d 11 (Figure 5). Seven bacterial genera where differences were apparent could be distinguished (Table 5). *Megamonas* spp. were more abundant in the CE groups compared to both control groups. *Eisenbergiella* spp. were more abundant in Cpos compared to CEs, while *Escherichia* spp. were more abundant in Cpos compared to both CE groups. Unclassified *Lachnospiraceae* spp. were more abundant in the Cneg group compared to all other groups. *Colidextribacter* and *Pseudoflavonifractor* spp. were both more abundant in the Cneg group compared to both control groups. *Clostridia vadinBB60* group had a higher abundance in the Cpos group compared to the CEw group.

The top 10 most dominant genera were present in a relative abundance ranging from 47.8% to 98.9% within

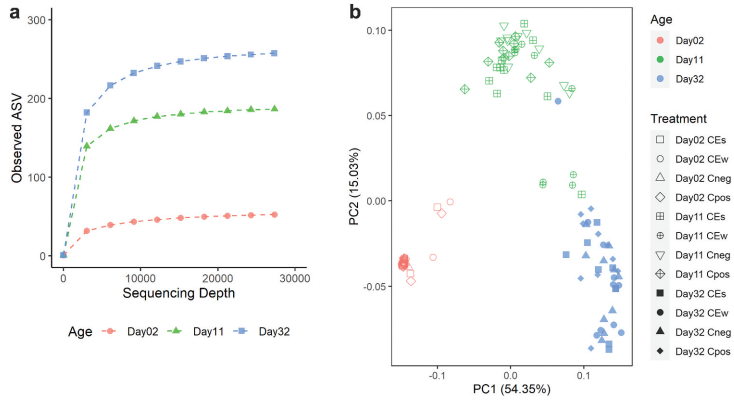

Figure 4. (a) Rarefaction curves of observed amplicon sequence variants (ASV) in caecal samples of different ages and (b) principal coordinate analysis (PCoA) plot showing differences in generalised UniFrac beta diversity at different treatments and ages. Treatments: chicks in the hatcher were given no access to feed and water (Cneg), access to feed and water (Cpos), access to feed, water, and a competitive exclusion (CE) product provided in the water (CEw), or access to feed, water and a CE product sprayed on the down of the newly hatched chicks (CEs). Different treatments are indicated by symbols, ages are indicated by colours.

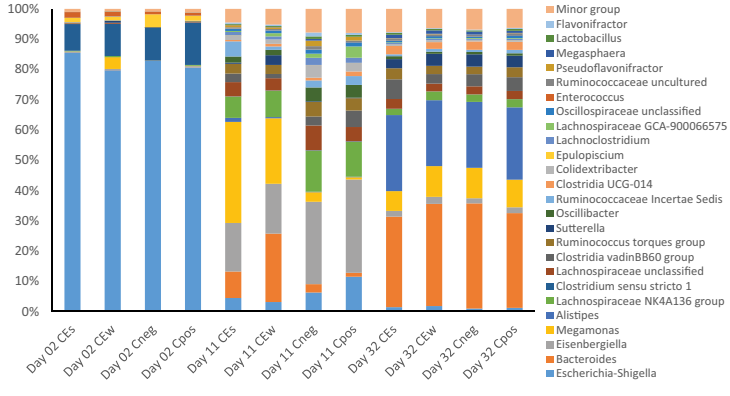

Figure 5. The relative abundance (%) of genera in caecal samples at three different ages (2, 11 and 32 d of age) in chickens given one of the following four treatments at hatch: chicks in the hatcher were given no access to feed and water (Cneg), access to feed and water (Cpos), access to feed, water, and a competitive exclusion (CE) product provided in the water (CEw), or access to feed, water and a CE product sprayed on the down of the newly hatched chicks (CEs).

Table 5. Estimated marginal means (± SE) of genus level sequencing counts differing between hatching treatments at d 11.

	CEs	CEw	Cneg	Cpos
Eisenbergiella	[1]8.38 ± 0.206[a2]	8.41 ± 0.203[ab]	8.91 ± 0.158[ab]	9.04 ± 0.148[b]
Megamonas	9.12 ± 0.149[b]	8.68 ± 0.186[b]	6.76 ± 0.486[a]	5.33 ± 0.992[a]
Escherichia-Shigella	7.1 ± 0.24[a]	6.74 ± 0.287[a]	7.44 ± 0.202[ab]	8.05 ± 0.149[b]
Unclassified *Lachnospiraceae*	7.16 ± 0.134[a]	7.01 ± 0.144[a]	7.71 ± 0.102[b]	7.16 ± 0.134[a]
Clostridia vadinBB60 group	6.66 ± 0.293[ab]	5.94 ± 0.42[a]	6.72 ± 0.284[ab]	7.3 ± 0.212[b]
Colidextribacter	6.04 ± 0.21[a]	5.95 ± 0.22[a]	7.04 ± 0.127[b]	6.63 ± 0.156[ab]
Pseudoflavonifractor	5.24 ± 0.308[a]	5.05 ± 0.34[a]	6.2 ± 0.191[b]	5.86 ± 0.226[ab]

[1]Values are estimated marginal means ± standard error. Results are given on the log scale.
[2]Values within rows lacking a common superscript are significantly different ($P < 0.05$).

Table 6. Mean relative abundance of the top 10 genera detected in caecal samples from chicks at 2, 11 and 32 d of age.

Genera	Day 2 (%)	SD	Day 11 (%)	SD	Day 32 (%)	SD
Escherichia-Shigella	82.14	7.59	6.30	5.85	1.33	1.68
Bacteroides	0.07	0.04	8.87	18.08	32.50	21.37
Eisenbergiella	0.27	0.94	22.64	13.88	1.96	1.23
Megamonas	1.07	4.44	14.74	18.65	9.04	6.39
Alistipes	0.00	0.00	0.52	1.38	23.10	11.94
Lachnospiraceae NK4A136 group	0.22	0.74	10.22	7.35	2.51	1.83
Clostridium sensu stricto 1	11.12	6.21	0.10	0.13	0.04	0.08
Unclassified *Lachnospiraceae*	0.16	0.42	5.42	2.61	2.79	0.94
Clostridia vadinBB60 group	0.02	0.06	3.17	3.33	4.49	4.01
Ruminococcus torques group	0.12	0.42	3.77	2.08	3.03	1.59

each sample (Table 6). The age-related change was clearly apparent in the most obvious microbial shift, where *Escherichia* spp. and *Clostridium sensu stricto 1* were the two most dominant genera at the beginning of the chick's life (d 2) and decreased considerably, to the advantage of other species, by d 11 and 32. In contrast, *Bacteroides* spp. presented at very low levels on d 2, but increased by d 11 and became the most dominant genus by d 32. *Alistipes* spp. were present at very low levels on both d 2 and 11, but became the second most dominant genus by d 32. *Eisenbergiella*, *Megamonas*, and *Lachnospiraceae NK4A136* spp. were present at low levels on d 2, became dominant by d 11, but eventually decreased in relative abundance by d 32. An unclassified *Lachnospiraceae* spp. together with *Clostridia vadinBB60* and *Ruminococcus torques* were all present at low levels on d 2 but increased by d 11 and maintained the same levels to d 32. Despite the general trend observed over the age of the birds, there was great variation in microbiota composition of individual birds within the same treatment group at same age.

Discussion

The aim of this study was to determine whether adapted management routines immediately post-hatch can improve the development of immune response and growth in broiler chicks. To the authors' knowledge, this is the first longitudinal scientific study providing results on a broad spectrum of variables such as immunological responses, organ development and productivity in chickens that have received feed, water and a CE product already in the hatcher. No effects of hatching treatments on antibody traits, gut microbiome development, organ development or intestinal morphology that lasted throughout the study were found. However, delayed access to feed and water reduced weight gain and feed intake early in the growth period. Physiological differences due to time to feed intake post-hatch have been investigated in many studies (Noy and Sklan 1999; Juul-Madsen et al. 2004; Van de Ven et al. 2013), and such disadvantages associated with prolonged time to feed access have been thoroughly reviewed (Willemsen et al. 2010;Powell et al. 2016).

In the present study, some effects were observed when the chicks were not allowed initial access to feed and water, mostly with regard to early organ growth and body weight gain. Early fed chicks generally prioritised the development of the gastrointestinal tract. In previous studies, increased length and weight of the ileum and jejunum have been observed in chicks fed early post-hatch (Maiorka et al. 2003). This corresponded with the findings in the present study, where, at d 2, feed-deprived chicks had both shorter and lighter intestines in relation to body weight and in absolute terms, than all other treatment groups. However, the intestines were weighed with digesta in this study, which may have biased the results due to *e.g.*, timing of sampling in relation to feed intake.

It has been reported that amino acids derived from yolk protein and most of the general energy in the yolk are spent on gastrointestinal development in fed and feed-deprived birds (Noy and Sklan 1999). The non-fed chicks in the present study tended to have lower relative gizzard weight when gizzards were not emptied and rinsed before weighing, which was logical due to the lack of feed. However, non-fed chicks had higher relative empty gizzard weight than all other groups. This suggested that non-fed chicks may have given priority to digestive organs located higher up in the digestive tract (*e.g.*, gizzard), to prepare for efficient feed digestion, and that they prioritised lower GIT development (small intestine) later, when feed was available. In agreement with our findings, in a meta-analysis, De Jong et al. (2017) found relatively shorter and lighter gut segments in the first week of life in feed- and water-restricted chicks. Conversely, Lamot et al. (2014) found proportionally longer intestines in feed-restricted chicks. However, De Jong et al. (2017) found lower villus height and crypt depth, particularly during the first week of life, which suggested that differences in organ development due to feed and water restriction ($\geq$36–60 h) may only be short-term. Therefore, sampling at 2 and 11 d in the present study might not have been optimal for detection

of differences in intestinal development, including morphology. Because differences in gut development seem to be highly dependent on sampling day in early life, which made comparisons between studies difficult (Ivarsson et al. 2022). It is likely that some differences in relative organ weight may be due to differences in body weight gain between treatment groups, and not organ development per se, which makes results difficult to compare between studies. However, in this study, differences in small intestine length and weight between fed and non-fed chicks were apparent when comparing absolute values.

At 2 d of age, the CEw group had significantly greater relative intestinal weight than both control groups, which indicated that the CE product supported intestinal development in early life. This effect did however not persist throughout the study and did not generate any other beneficial effects. Similarly, O'Dea et al. (2006) did not find any differences at the end of the grow-out period regarding body weight, feed conversion, or mortality in chicks provided with probiotics through four different administration routes at hatch. Relative weight of intestine, heart, liver, proventriculus, and gizzard decreased with age in this study, as did relative length of intestine, corresponding well with previous findings (Boyner et al. 2020; Ivarsson et al. 2022). Relative weights of spleen and bursa increased with age, which agreed with Kaiser and Balic's (2014) description of the bursa reaching its maximum size at approximately eight weeks of age and thereafter regenerating. The relative weight of the bursa was greater at 20 d of age than at 6, 10, and 34 d of age in the study by Boyner et al. (2020), whereas there was no effect of ageing with regard to bursal weight in the study by Ivarsson et al. (2022). However, the latter observed that relative spleen weight increased with age (Ivarsson et al. 2022), as found in the present study.

Unsurprisingly, the Cneg group experienced a disadvantage in body weight gain compared with all other groups in early life (d 2, 4, and 11). Lower body weight has been shown to persist for up to six weeks in chicks kept feed- and water-restricted for 48 h (De Jong et al. 2017). However, this was not the case in the present study, possibly because the chicks were only subjected to feed and water restriction for 40 h. Unfortunately, all treatments were constrained by lack of feed and water during transportation. These conditions were probably not in favour of GIT development or other traits, which may have made the results less comparable to those in other studies. Another risk of withdrawal of feed and water during transportation after it has been offered is that a slightly more developed intestine (as in the case with the CEw group) can signal hunger, which may cause the chickens more stress during transport.

Vertical transmission of gut microbiota from the mother hen to her offspring via the oviduct (Shterzer et al. 2020) is a mechanism facilitated by the embedding of microbiota (beneficial or pathogenic) in the developing egg and has been known for some time. In addition, under natural conditions, the hatching chick comes into contact with environmental and conspecific microbes already when its egg tooth hits the shell. In terms of microbiota development, the chick would likely benefit from close contact with the hen, gaining a commensal healthy microbiota. In modern production systems, this natural step in microbial transfer is not available to the chicks, making them more vulnerable to possible pathogenic microbes colonising their gut instead (Carrasco et al. 2019). However, some phyla of microbiota important to the chick have been discovered which are not primarily obtained from the mother hen. When chicks were hatched together with a hen in one study, donor hens did not seem sufficient as a source of *Firmicutes* spp. (Kubasova et al. 2019). This suggests that *e.g.*, *Lachnospiraceae* and *Ruminococcaceae* spp. originate from the surrounding environment rather than from adult birds (Kubasova et al. 2019). On the other hand, the *Firmicutes* phylum has been highlighted as one of the most easily transmitted phyla between hen and offspring (Aruwa et al. 2021).

In the present study, *Megamonas* spp of the phylum *Firmicutes* was the only genus more abundant in the CE groups compared to both control groups at 11 d of age. Moreover, *Firmicutes* is the second most abundant phylum in Broilact® (Such et al. 2021). *Megamonas* spp. has been speculated to be highly abundant in chicken caeca due to its hydrogen removing capacity, which is thought to benefit other microbes. This might have an indirect beneficial effect on the host, by improved energy recovery from feed (Sergeant et al. 2014). Unclassified *Lachnospiraceae*, *Colidextribacter* and *Pseudoflavonifractor* spp., all members of *Firmicutes* phylum, were all significantly more abundant in the Cneg group compared to CE groups. One explanation for this could be that these three genera were unable to compete in the same place as *Megamonas* spp., hence they could increase in the Cneg group instead. However, why *Colidextribacter* and *Pseudoflavonifractor* spp, were not more abundant in the Cpos group remained unclear. Although being the most abundant genus in Broilact®, *Escherichia* spp. was more abundant in the Cpos group compared to both CE groups. As concluded by Ballou et al. (2016), addition of bacterial cultures, such as probiotics, when chickens are reared under non-stressful conditions seem only to have small or transient effects on the microbiome's function and activity. However, the same authors highlighted the important effect of age on the microbial composition and diversity and pointed out age-dependent shifts in dominant phyla during the chicks' life, as reported in the current study.

The rarefaction curves of ASVs in the present study clearly demonstrated increased richness in microbiota with age (Figure 4(a)). Increased caecal microbiota richness and diversity with increasing age has been reported in many previous studies (Oakley et al. 2014; Ballou et al. 2016). The microbiota of the young chick typically has low diversity and is dominated by *Enterobacteriaceae* spp. (Ballou et al. 2016). Microbial diversity starts to increase around 7 d of age, when the phylum *Firmicutes* increases in abundance (Ballou et al. 2016). This corresponded well with findings in the present study on relative abundance of the top 10 genera, where *Escherichia* spp. (*Enterobacteriaceae*) was dominant at 2 d of age. Moreover, there was an obvious shift towards genera within the *Firmicutes* phylum at 11 d of age (*Clostridium sensu stricto 1* being the exception), which agreed with previous findings (Kubasova et al. 2019). Overgrowth of *Clostridium sensu stricto 1* in combination with a decrease in *Lactobacillus* spp. in the jejunum has been correlated with the development of necrotic enteritis in chickens (Yang et al. 2019). At approximately four weeks of age, there is another compositional shift where *Firmicutes* spp. are generally accompanied by *Bacteroidetes* spp. (Kubasova et al. 2019). This was the case in the present

study, where the genera *Bacteroides* and *Alstipes* spp. showed higher abundance at 32 d of age. Development of the caecal microbiota seemed to follow the normal maturation pattern in this study, with corresponding shifts in ageing modern broiler caecum, and differences in microbial composition between hatching treatment groups at d 11 were no longer apparent at d 32. The inferior FCR observed in the CEw group was therefore presumably not due to any microbiota-related differences. However, in a study by Such et al. (2021), some changes in microbiota composition due to Broilact® were observed in chicks at 7 d of age, but not at later time points It was concluded that differences in microbiota composition are determined mostly by sampling site and time point (Such et al. 2021). On the other hand, Broilact® treatment has been found to increase resistance towards colonisation of *Salmonella enterica* by competitive exclusion (Schneitz et al. 2016). In a field study, Broilact® supplementation was associated with positive, but non-significant effects on *Clostridium perfringens*-associated lesions and performance traits (Kaldhusdal et al. 2001). In the present study, individual water consumption was not recorded and chicks in the CEs group were sprayed manually with a handheld spray bottle, so it was possible that the Broilact® solution was unevenly consumed by the chicks. Moreover, under commercial settings, Broilact® is not sprayed manually, but in an automatic cabinet. These circumstances may have affected the results obtained for intestinal microbiota.

In order to assess responses to a novel antigen, the birds in the present study were vaccinated with an inactivated virus vaccine. As the read-out for the vaccine-induced immune responses, antibody production to APV was used. However, only 44% of the birds developed antibodies to APV and birds that tested positive generally had low antibody levels to this antigen. Hence, it was difficult to identify any putative effects of the experimental treatments on this trait. This low responsiveness to vaccination was unexpected and no clear explanation was identified. In Sweden, broiler chickens are not routinely vaccinated post-hatch, but, internationally, broiler-type chickens are regularly subjected to vaccination programs comprising vaccines against several infectious diseases (Sharma 1999; Landman 2012). The vaccines used in these programs are often live, which are generally considered more potent as immune activators (Aida et al. 2021). Thus, a live vaccine might have induced more prominent antibody responses in the birds in this study. However, some inactivated vaccines are used for broilers (Sharma 1999) and Juul-Madsen et al. (2004) observed clear antibody responses in Ross 208 chickens after administration of an inactivated vaccine against infectious bursal disease virus at 10 d of age. Hence, it seems unlikely that the choice of an inactivated vaccine was the sole reason for the poor responses in the present study. Genetic background has an influence on immune response and it has been shown that antibody production upon immunisation may be influenced by selective breeding of chickens (Minozzi et al. 2008; Zerjal et al. 2021).

In the present study there was a correlation between birds responding to the APV vaccination and higher serum levels of total IgY at d 31. This indicated that chickens responding to the vaccination also produced more antibodies in general, *i.e.*, could potentially be identified as high antibody responders, which suggested that antibody production may have been influenced by genetic factors in the experimental birds. Moreover, concerns have been raised that the modern broiler chicken may have generally low immune responsiveness due to potentially heavily biased selection for increased growth (Van der Most et al. 2011), which may have contributed to the poor vaccine-induced responses observed in the present study. Consequently, it seemed likely that several factors contributed to the observed low vaccine-induced antibody responses.

Other factors that may have contributed to the relatively low immune responsiveness and limited effects of the experimental treatments may have included the low stocking density during the experiment and high biosecurity at the research facility. The university research facility used in this study may not have provided the same challenge to the birds' immune system as those encountered under commercial conditions, where more birds are kept in the same pen at higher stocking densities. Moreover, the research facility has no birds for long periods between studies, which may have resulted in lower pathogenic pressure compared to a commercial set-up. This hypothesis was supported by findings reported by Eckert et al. (2010), who did not detect any differences in body weight or FCR in chickens provided with probiotics in the drinking water until the stocking density was increased to simulate commercial conditions. In fact, those researchers had to almost double the number of chickens in the rearing facility before they observed increased body weight and lowered FCR in probiotic-fed birds (Eckert et al. 2010).

A more potent infectious or inflammatory challenge to the chickens in the present study might have revealed greater impacts of the experimental treatments. In a study by Van den Brand et al. (2009) where the challenge was a cocktail of lipopolysaccharide/human serum albumen (as a model for lung infection), chickens that were kept for 24, 48 or 72 h post-hatch without access to feed and water showed significantly lower body weight gain post-challenge than birds fed directly post-hatch. Those authors concluded that directly fed birds can withstand immunological challenges better, although this was not confirmed by the mean values of different antibody titers (Van den Brand et al. 2009).

In the present study, early measures were taken in the hatcher, in terms of provision of feed, water and a CE product, to strengthen the immune response and prerequisites for growth of the newly hatched chicks. For the observed early differences between hatching groups, only the difference in feed intake between control groups and the increased FCR in CEw birds persisted throughout the study. Moreover, no new differences appeared, which suggested that modern broiler chickens are capable of compensating for 40 h of feed and water deprival post-hatch.

Provision of Broilact® did not have any persistent performance-enhancing properties with the set-up tested, although an experimental set-up allowing chicks to continue their respective treatments (especially water access for relevant groups) during transportation might have given a different outcome. As mentioned by Ballou et al. (2016), probiotics may have only small effects on the microbiome when chickens are reared under non-stressful conditions. An experimental environment closer to that in commercial production, mimicking more fairly the pathogen pressure

and stocking densities, might also have given a different outcome.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Conventional hatching practices do not involve provision of feed and water to broiler chickens before placement at the rearing farm. In this thesis, hatching concepts allowing post-hatch feeding were studied. The results suggest modern broiler chickens are at least partly capable of compensating for setbacks in early life. Further research is however needed to confirm the effects of adapted hatching and post-hatch feeding strategies in conditions that resemble more closely the challenges faced by the modern broiler.

Malin Boyner received her postgraduate education at the Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences. She received her Master of Science degree in Agriculture (Animal Science) at the same university.

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