Transmission dynamics of *Eimeria acervulina*

in broilers

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 10 June 2011
at 1.30 p.m. in the Aula

Voor mijn moeder

Abstract

Velkers, F.C. (2011). Transmission dynamics of *Eimeria acervulina* in broilers.
PhD Thesis Wageningen University, the Netherlands.

Control of the intestinal disease coccidiosis, caused by infections with *Eimeria* species, is a major challenge, especially for the broiler industry. Effective control strategies require a comprehensive understanding of processes that lead to infection and disease in a population. One of the key factors that determine infection dynamics in a flock is the rate of transmission between hosts. Therefore, transmission experiments were carried out to increase the understanding of the underlying mechanisms of *Eimeria acervulina* infections in broilers, to facilitate improvement of control strategies. An important outcome of the experiments was that the excreted oocyst dose, which may be related to severity of clinical signs, increased during successive generations of infection in the flock, but that the transmission rate was independent of the oocyst dose. This suggests that transmission is not determined by the number of oocysts excreted with faeces of infected birds but, most likely, by the probability of birds to come into contact with infectious faeces. Factors influencing the degree and dispersal of infectious faecal material in the environment, such as movements and (litter pecking) behaviour of chickens, environmental conditions and faeces characteristics, may have a large impact on infection dynamics and efficacy of control measures. Furthermore, it was demonstrated that a previous infection with a wild-type *E. acervulina* strain significantly reduced oocyst output and transmission after re-infection. After infection with a live vaccine strain, oocyst output following an infection with a wild-type strain was also significantly reduced. However, a significant reduction of transmission of the wild-type strain was not found in groups of broilers that had been infected with the vaccine strain. Nevertheless, it was demonstrated that the live vaccine was efficiently transmitted to initially unvaccinated birds. Furthermore, the level of reduction of oocyst output was equally high for directly vaccinated and the "contact-vaccinated" chickens, that became infected due to ingestion of oocysts excreted by vaccinated birds. These results indicate that transmission of the vaccine can induce protection against high oocyst output for the entire flock, even when not all birds receive the vaccine during the initial mass application. The results of these experiments indicate that influencing the rate of transmission of wild-type and vaccine strains can be important for reducing the adverse effects of flock infections with *Eimeria*. Furthermore, this thesis has increased insight into some of the underlying factors that determine transmission dynamics of *E. acervulina* in a broiler flock. Further investigation of these factors may reveal novel targets or facilitate improvement of current strategies for coccidiosis control.

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Chapter 1

General Introduction

1.1. Coccidiosis in chickens

Coccidiosis in chickens is an intestinal disease caused by protozoan parasites of the genus *Eimeria*, belonging to the taxonomic family Eimeriidae (Levine, 1982) and the phylum Apicomplexa (Levine, 1970). Other protozoa in this phylum, causing disease in humans and animals, include species of *Isospora*, *Babesia*, *Atoxoplasma*, *Toxoplasma*, *Neospora*, *Cryptosporidium* and *Plasmodium* (Levine, 1985; Shirley et al., 2005).

Nine species of *Eimeria* have been described in chickens, of which at least seven species are relevant for the poultry industry, i.e. *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria praecox*, *Eimeria necatrix* and *Eimeria tenella* (McDougald, 2008). The most frequently diagnosed species in intensively reared poultry include *E. maxima*, *E. tenella* and the most prevalent one, *E. acervulina* (Williams et al., 1996). The control of these three *Eimeria* species is usually accorded a higher priority over the other four species, especially in broiler chickens (McDougald et al., 1997; Shirley et al., 2005).

Eimeria parasites multiply in epithelial cells of the intestinal tract, causing tissue damage and changes in intestinal function. Coccidiosis can be characterised by apathy, loss of appetite, ruffled feathers, huddling, mortality, diarrhoea, bloody faeces and reductions in weight gain and efficiency of feed conversion (Williams, 1999; Dalloul and Lillehoj, 2005; McDougald, 2008; McDonald and Shirley, 2009).

Coccidiosis is the most frequently reported disease in poultry. With about 40 billion chickens raised annually worldwide, the disease is estimated to cost around 800 million US dollars per annum, due to production losses and costs for (prophylactic) intervention (Williams, 1999; Sharman et al., 2010).

1.2. Characteristics of *Eimeria* infections in broiler chickens

The infective form of *Eimeria* is the oocyst, which is shed with the faeces of infected birds. The oocyst is excreted from the host as an undifferentiated stage and becomes infective after sporulation, which mainly depends on temperature, humidity, and oxygen (Kheysin et al., 1972; Waldenstedt et al., 2001). The sporulated oocyst contains four sporocysts and each sporocyst contains two sporozoites. Infection occurs via a faecal-oral route. A susceptible chicken becomes infected when it orally ingests one or more sporulated oocysts from the environment. The oocyst wall is crushed in the gizzard and sporozoites are released from the sporocysts in the small intestine under the influence of chymotrypsin and bile salts and enter epithelial cells. Three or four rounds of asexual reproduction (schizogony) are followed by sexual differentiation (gametogony), fertilisation and, finally, shedding of the unsporulated oocysts (Levine, 1985; Shirley et al., 2005). The prepatent period, the time between ingestion of sporulated oocysts and the appearance of the first oocysts in the faeces, is 4-7 days, depending on the *Eimeria* species (Edgar, 1955; Joyner and Long, 1974; McDougald, 2008).

An important feature of *Eimeria* species is its high reproductive potential. One oocyst ingested by a chicken can result in an offspring of thousands to several hundred thousand oocysts and a single infected bird can produce several millions of oocysts (Williams, 2001; McDougald, 2008), contaminating the litter of the poultry house. In the absence of re-infection, infections are self-limiting and infected birds usually shed oocysts for several days or weeks during the patent period. After five days in the litter, oocysts start to disintegrate, presumably due to the presence of bacteria and ammonia, and viability of the oocyst population begins to wane after about three weeks (Reyna et al., 1983; Williams, 1995). As a result of repeated ingestion and excretion of new progenies of the parasite, viable oocysts can be detected in the faecal droppings and litter in a flock for several months after the initial flock infection (Williams, 1998; McDougald, 2008).

As most poultry houses are cleaned between subsequent flocks, only small numbers of oocysts are present when a new flock of chickens arrives. It is therefore assumed that the flock infection starts with a small number of chickens that ingest a low dose of oocysts (Long et al., 1975). The high reproduction potential of the parasite and infection of other birds subsequently results in a quick increase of the oocyst load in the environment (Shirley et al., 2005).

1.3. Control of coccidiosis

Oocysts are ubiquitous, easily dispersed in the poultry house environment, very resistant to disinfectants and have a large reproduction potential. It is therefore difficult to prevent infection in flocks, despite the use of stringent hygienic measures (Long, 1984; Reid, 1989; Allen and Fetterer, 2002; McDougald, 2008). Consequently, commercial flocks free from coccidia are extremely rare (Williams et al., 1999). Although good husbandry techniques are important, additional measures are necessary to control the disease. Since the late 1940s, prophylactic chemotherapy with anticoccidial drugs given in the feed has been the primary control measure against coccidiosis. This abundant use of medication, however, has resulted in emergence of drug resistance against most anticoccidials (McDonald and Shirley, 2009; Sharman et al., 2010). Furthermore, an increasing concern regarding drug residues in poultry products has resulted in bans by European Union governments for several anticoccidial drugs and plans of the European Commission to completely phase-out the use of these drugs by 2012 (European Community, 2003; Shirley et al., 2007).

These developments have propelled the search for alternative control strategies for coccidiosis. Coccidia are highly immunogenic and a primary infection can stimulate solid immunity to infection with a homologous species. Therefore, vaccination could provide an excellent alternative to chemotherapeutics for controlling coccidiosis (Allen and Fetterer, 2002). Live attenuated or non-attenuated vaccines are frequently used in layer and breeder flocks, but the use in broilers remains limited. Live vaccines are costly to produce and have a

short shelf life. Moreover, due to the short life span of broilers, the time to develop protective immunity against field strains of *Eimeria* and the time for compensatory growth after a transient vaccine induced growth reduction is short. Another concern is that due to mass application of vaccine, which is necessary in broiler flocks, optimal uptake of vaccine for all birds in the flock is presumably not always achieved. This might cause insufficient vaccine-induced protection of the flock against infections with wild-type strains and, when non-attenuated strains are used, can lead to outbreaks with vaccinal oocysts (Chapman et al., 2002; Williams, 2002; Shirley et al., 2005). Vaccination is therefore often too expensive for broiler producers, due to the small profit margins in this industry. To reduce costs, some broiler farms with high losses due to clinical coccidiosis vaccinate a few subsequent flocks, in an attempt to restore drug sensitivity of the resident parasite population, followed by a few rounds with anticoccidials (Peek, 2010; Shirley et al., 2005).

Recently, the focus of coccidiosis research has been towards alternatives to live vaccines to overcome some of the drawbacks of live vaccines, such as recombinant protein/subunit or DNA vaccines (Dalloul and Lillehoj, 2006), *in ovo* vaccination (Weber et al., 2004; Ding et al., 2005) and use of immunomodulators, such as cytokines (Dalloul and Lillehoj, 2005). Of these alternatives, only the subunit vaccine CoxAbic®, a vaccine developed to induce protection of offspring by maternally derived antibodies (Wallach, 2010) is currently available for commercial use. Furthermore, herbs, essential oils, probiotics, prebiotics and changes in feed composition and structure, are studied and used increasingly, but anticoccidial activity has not been demonstrated unambiguously (Allen and Fetterer, 2002; Peek, 2010).

1.4. Host-parasite interactions and infection dynamics

An infection with *Eimeria* species causes a strong, species-specific protective immunity, which is stimulated by the initial developing parasite stages, particularly the schizonts (Sharman et al., 2010). The interaction between the parasite and the immune response of the host is complex and is determined by factors such as host genetic background, nutritional status of the host, parasite species/strain and the infection history of the host (Rose and Long, 1962; Joyner and Norton, 1976; Lillehoj, 1988; Shirley et al., 2005). Uptake of few oocysts can potentially establish an infection (Norton and Joyner, 1986), but a higher dose of oocysts generally results in a higher oocyst yield in infected birds, until a certain infection dose is reached, the "crowding threshold" (Williams, 2001). Protection against re-infection tends to improve with increasing oocyst dose (Hein, 1976; Rose, 1987; Lillehoj, 1988; Blake et al., 2005) and repeated infections can boost and maintain protective immunity (Hein, 1975; Joyner and Norton, 1976; Galmes et al., 1991; Stiff and Bafundo, 1993; Chapman and Cherry, 1997; Blake et al., 2005; Velkers et al., 2010).

These interactions between *Eimeria* and the individual host can have a large impact on infection dynamics in a flock and have been the main focus of many studies (Williams and

Catchpole, 2000; Chapman et al., 2002; Holdsworth et al., 2004; Chapman et al., 2005). Although valuable knowledge on host-parasite interactions and pathogenesis of the disease has been gained, one of the most important characteristics of an infectious agent, i.e. the ability to spread from one animal to another, has hardly been included in studies so far. Transmission of an infectious agent depends on many different factors, such as the infectivity of infected birds, susceptibility of not yet infected birds, survival and distribution of infectious material in the environment and behaviour of birds. Although either related to the host, the parasite, the environment or the population, all of these factors interact and mutually affect each other and collectively determine the dynamics of the infection in the flock and as a result, affect performance and disease of the flock (Roberts and Heesterbeek, 1995; Graat et al., 1996; Roberts and Heesterbeek, 1998; Klinkenberg and Heesterbeek, 2005; Severins et al., 2007; Grassly and Fraser, 2008).

The number of oocysts excreted by an infected bird, for instance, is influenced by interactions between the individual host and the parasite. The response of the immune system of the host, however, is also affected by the previous oocyst exposure history of the host and the ingested oocyst dose from the environment, which in turn are both a result of excretion of oocysts by other birds during the course of the flock infection. Consequently, in addition to interactions between hosts and the parasite, aspects regarding population-dynamics should be determined as well when studying efficacy of control strategies.

Transmission experiments can be used to study these dynamical aspects of an infection. Furthermore, with the aid of mathematical models, such as the *SI* (susceptible-infectious) model, the data from these experiments can be used to quantify transmission characteristics of a pathogen (Becker, 1989; Louz et al., 2010). For *Eimeria* species, however, transmission experiments have not been carried out previously and other experimental studies describing infection dynamics for *Eimeria* species are scarce. A study by Graat et al. (1996), using a previously developed mathematical model (Henken et al., 1994) and field data, has shown that flocks exposed to an intermediate initial contamination level showed better production performance than broilers exposed to either high or low levels of oocysts in the environment. Furthermore, mathematical models, describing relations between oocyst input, oocyst output and immunity, have shown that infection dynamics can be complex and that the outcome of interactions between within-host and between-host dynamics may give rise to a counter-intuitive and an unpredictable course of the infection (Parry et al., 1992; Henken et al., 1994; Graat et al., 1996; Klinkenberg and Heesterbeek, 2005; Klinkenberg and Heesterbeek, 2007; Severins et al., 2007). Experimental data from transmission experiments, complemented with the use of mathematical models and field studies, are important to unravel important factors that determine transmission dynamics, which can facilitate the design and optimisation of intervention strategies. In this thesis, transmission experiments in pairs and groups of birds are described to contribute to the knowledge of infection dynamics of *Eimeria*.

1.5. Scope and outline of this thesis

The main objective of this thesis was to obtain insight into transmission dynamics of
E. acervulina in broiler flocks to improve efforts to control coccidiosis. Important tools for
studying the transmission dynamics, i.e. experimental and mathematical methods to quantify
transmission and to adequately determine oocyst output in individual birds, were developed
and validated.

Important factors determining the course of infection in a flock are the numbers of
ingested and excreted oocysts and transmission characteristics of the parasite. For
quantification of transmission of the parasite, it is necessary to determine for individual birds
if and when a bird has become infected by examining the faeces for the presence of oocysts.
Furthermore, quantification of the number of oocysts in faeces of individual birds can be
important to determine oocyst output patterns, and may give information on the ingested
dose, the severity of infection in individual birds and on the contribution of individual birds to
the oocyst load for other birds. In *chapter 2*, two methods are described to determine oocyst
excretion for individual birds, i.e. using oocyst counts on single individual faecal droppings or
a quantitative PCR on cloacal swabs. For validation of both procedures, oocyst counts and
quantitative PCR results were compared to the gold standard of oocysts counts for 24 h faecal
samples.

In *chapter 3*, the single dropping procedure was applied in a pair-wise transmission
experiment, where one of the birds was inoculated with *E. acervulina* oocysts and the other
bird was contact-exposed. Four different inoculation doses, ranging from a very low dose of 5
oocysts to a high dose of 50,000 oocysts, were used to study the relation between the
ingestion of oocysts (inoculation dose), oocyst excretion and transmission.

A pair-wise setup was also used for a combined immuno-epidemiological study (*chapter 4*).
By using different combinations of birds, consisting of either previously exposed or naïve
birds, effects of a previous infection history with *E. acervulina* could be linked to oocyst
output and transmission between birds and the local immune response in the intestinal tract.

In *chapter 5*, transmission was studied in a group of 20 birds. It was studied whether an
increase in oocyst output occurred for successive generations of infection during a flock
infection and whether this affected the transmission rate parameter.

In *chapter 6*, live vaccination was studied in a transmission experiment with groups of
birds. Mass application of live vaccines might not result in uniformity of exposure to vaccine
oocysts in the flock and might reduce efficacy. Therefore, experiments were carried out to
study the effects of vaccinating part of the flock on transmission and oocyst excretion of a
vaccine strain of *E. acervulina*, and on transmission and excretion after a subsequent
challenge infection with a wild-type *E. acervulina* strain for unvaccinated, vaccinated or
contact-vaccinated birds (infected with vaccine oocysts due to exposure to vaccinated birds).

In the general discussion, the findings of these experiments and implications for (future
research on) transmission dynamics and control of coccidiosis are discussed.

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Chapter 2

Quantification of *Eimeria acervulina* in faeces of broilers: Comparison of McMaster oocyst counts from 24 h faecal collections and single droppings to real-time PCR from cloacal swabs

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Abstract - Coccidiosis is an economically important disease in chickens, caused by infection with *Eimeria* species parasites. Diagnosis of coccidiosis is frequently based on oocyst enumeration in pooled faecal samples or litter. In studies on infection dynamics and for monitoring in the field, samples from individual chickens may be more appropriate as these support the determination of infection status of individual birds and more accurately reflect oocyst output at time of sampling. Faecal samples from individual birds can be collected, but the counting procedure limits the number of samples that can be processed and unequivocal microscopic differentiation between *Eimeria* species is very difficult. A test that overcomes these drawbacks would improve efficiency and quality of the diagnosis. The aim of this study was to compare two methods for *Eimeria* oocyst quantification in samples from individual birds. A real-time PCR that quantifies oocysts in cloacal swabs (qPCR) and oocyst counts in single droppings were compared to the standard procedure of oocyst counts in bulked 24 h faeces. Faecal samples were collected daily from 30 broiler chickens, inoculated with different doses of *Eimeria acervulina*. The three techniques produced comparable oocyst counts for all inoculation doses. Single dropping counts are applicable for small sample sizes and when a single *Eimeria* species is used. For larger sample sizes qPCR is preferable as it can be carried out on samples that have been frozen for storage. Furthermore, qPCR can identify and quantify different *Eimeria* species, which makes it a valuable diagnostic tool for field or experimental work.

Keywords - *Eimeria acervulina*; oocyst counts; quantitative real-time PCR; single droppings

1. Introduction

Coccidiosis, caused by the *Eimeria* species, is one of the most common intestinal diseases of poultry with major economic impact worldwide (Shirley et al., 2005). Current control measures rely mostly on the use of anticoccidials, supplemented with live attenuated vaccines. Increasing resistance among *Eimeria* species (Chapman, 1997; Peek and Landman, 2003), consumers objections and legislative restrictions against dietary supplementation with anticoccidials undermine the use of chemotherapy whilst the uptake of live vaccines remains limited, largely due to high production costs, stimulating demand for the development and evaluation of new control strategies for control of coccidiosis in the future. Improved diagnostic tools can be beneficial for experimental studies and monitoring of *Eimeria* infections in the field and consequently support progress in the control of coccidiosis (Morris and Gasser, 2006; Blake et al., 2008; Morgan et al., 2009).

Oocyst enumeration is one of the most widely used parameters of eimerian infection in chickens (Holdsworth et al., 2004). Usually, bulked faecal droppings or litter samples are collected for oocyst quantification (Long et al., 1976; Williams, 2001; Peek and Landman, 2006), which gives an indication of the oocyst build up over a certain time interval (Long and Rowell, 1975; Williams et al., 2000) but does not provide any information about the contribution of individual birds to the oocyst load. Furthermore, oocysts in such samples can rapidly be degraded depending on environmental conditions (Williams, 1995a, 2006) which may result in an underestimation of the number of oocysts in the faecal sample. To study the dynamics of eimerian infection in field populations the ability to use samples that accurately reflect individual bird oocyst output will be invaluable. Applications include repeated sampling of birds given a specific treatment, exhibiting a phenotype of interest or selected for regular monitoring throughout the course of an infection or a flock cycle and transmission experiments. Oocyst counts from single droppings, obtained from individual birds, has previously been proposed for this purpose (Hodgson, 1970). The collection of such samples from defined individuals, however, can be time consuming and the number of examined samples must be adequate for evaluating efficacy of an intervention method or field monitoring (Holdsworth et al., 2004).

Identification of *Eimeria* species based on oocyst morphology (Morris and Gasser, 2006; Blake et al., 2008), pre-patent period or site and extent of intestinal lesions is unreliable since different *Eimeria* species have overlapping properties (Long and Joyner, 1984). Methods that can overcome these drawbacks include quantitative real-time PCR (qPCR) on individual samples that can identify and quantify *Eimeria* species. Samples destined for qPCR quantification can be tested even after long periods in storage.

Non-quantitative PCR assays designed to speciate *Eimeria* species have been available for more than 10 years (Tsuji et al., 1997; Schnitzler et al., 1998; Fernandez et al., 2003; Jenkins et al., 2006; Haug et al., 2008). The more recent development of qPCR techniques, applicable with *in vivo* excised tissue, faecal samples and purified parasite suspensions, now provides a

powerful diagnostic tool (Blake et al., 2006; Morris and Gasser, 2006; Swinkels et al., 2006; Blake et al., 2008; Morgan et al., 2009). The objective of this study was to determine whether two oocyst quantification techniques, i.e. oocyst counts from single droppings and qPCR quantification of oocysts on cloacal swabs, are accurate, reproducible and practically applicable measures to quantify eimerian infection in individual chickens.

2. Materials and methods

2.1. Chickens and management
Thirty male Cobb/Hybro/Ross crossbred broiler chicks were obtained from the specific pathogen free parent flock of GD-Animal Health Service (Deventer, the Netherlands). All birds were tagged for identification at day of hatch (D0) and housed in groups of 15 birds. From D6 onwards birds were individually housed in stainless steel wired floor cages. Room temperature was gradually decreased from 33 °C at D0 to approximately 21 °C at D27 and a lighting scheme of 23 hours of light per day was used. Drinking water and a broiler starter ration (2900 kcal/kg of metabolisable energy and 21.6% crude protein) without anticoccidial drugs were offered *ad libitum*.

The birds were observed twice per day for signs of illness or welfare impairment and were housed, handled and treated following approval by the Animal Experimental Committee of Wageningen University (Wageningen, the Netherlands), in accordance with the Dutch law on experimental animals. All chickens were killed at D27 by cervical dislocation.

2.2. Parasites: preparation and inoculation
A suspension of sporulated *Eimeria acervulina* oocysts, stored at 6 °C in 2.5% (w/v) potassium dichromate, originating from a frequently rejuvenated *E. acervulina* reference laboratory strain (Weybridge W119, Central Veterinary Laboratory, Weybridge, 1990), was diluted in tap water to obtain inocula with 50,000, 500 and 50 sporulated oocysts per 1.1 ml. Oocyst number and sporulation percentage was determined microscopically using a Fuchs-Rosenthal haemocytometer counting chamber.

At D6, chicks were randomly assigned to five groups and were inoculated directly into the crop with 1.1 ml of the inoculum (Groups 2-5) or sham-inoculated with tap water (Group 1, control group). Birds in Groups 2-4 received a single dose of 50, 500 or 50,000 sporulated *E. acervulina* oocysts respectively and chickens in Group 5 received a trickle infection with daily doses of 50 oocysts from D6 to D15.

2.3. Collection of faecal samples
Faecal droppings from all individual birds were collected daily from D10 to D27. Three procedures were used to collect faeces, as explained below.

Procedure 1   Collection of "24 h faeces": a clean plastic sheet was placed beneath the wired cage floor for 24 hours. All faecal droppings on the sheet were collected and weighed. The faeces were subsequently mixed and 4 g was taken for further processing.

Procedure 2   Collection of "single droppings": each chick was placed in a cardboard box, lined with clean water resistant bitumen paper, for approximately one hour. Most chicks produced a dropping during this period. The dropping was collected in a tube, and weighed. Faecal samples collected using procedures 1 and 2 were stored at 4 °C prior to further processing within the same day.

Procedure 3   Cloacal swabbing of individual birds for real-time PCR quantification: swabs were taken when the birds were handled for placement in the cardboard box for procedure 2. All swabs were weighed before and after sampling and were frozen on the day of collection at – 20 °C. Samples were frozen in the year 2004 and were defrosted and used for real-time PCR quantification in 2007 and 2008.

2.4. McMaster oocyst counts from faecal samples

To quantify oocyst production, a modification of a McMaster counting chamber technique described by Long and Rowell (1958) was used. Briefly, 20 ml of a sodium chloride solution (151 g/l, specific gravity 1.1) was added to the 24 h faecal sample or single dropping. After homogenisation, 2 ml of the suspension was transferred into a centrifuge tube filled with 8 ml of a saturated sodium chloride solution (311 g/l, specific gravity 1.2). This suspension was used to fill two McMaster counting chambers (2 x 0.15 ml). Further 10-fold dilutions were made if more than 300 oocysts were present per chamber. The number of oocysts per g of faeces (OPG) was calculated: 333.3 *ab* / sample weight (*a* is the total number of oocysts; *b* is the dilution). The detection limit when *a* = 1 using this modified McMaster technique is 83 OPG for a 4 g faecal sample.

2.5. Real-time PCR quantification from cloacal swab samples

2.5.1. DNA dilution series

Sporulated *E. acervulina* oocysts were used as the basis of four 10-fold serial dilution series during the developmental phase of this study. Initially, genomic DNA was purified from sporulated *E. acervulina* oocysts and used to produce a series representing 10^0 to 10^6 *E. acervulina* oocyst equivalents per 5 μl in triplicate as described previously (Series A, assuming an average of 6.2 genomes per oocyst in the test sample given an overall sporulation rate of 70%; 33 μg/ml glycogen used as a carrier; Blake et al., 2008). Complementary Series B-D, each representing 10^0 to 10^6 *E. acervulina* oocysts, were created in triplicate using intact oocysts (70% sporulation rate). Series B: pure oocysts in sterile milli-Q water [i.e. molecular biology-grade water]; C: 100 mg faeces collected from uninfected SPF chickens, spiked with pure oocysts in milli-Q water, incubated on ice in 10% sodium hypochlorite for 10 min and washed

twice in sterile milli-Q water; D: as C without the sodium hypochlorite and subsequent wash steps. All samples in Series B-D were pelleted in 2 ml screw-cap tubes (1 min, 10,000 x g) and smashed using a Mini-BeadBeater-8 for 2 min at "homogenise" (Biospec Products, Bartlesville, USA; as described previously; Blake et al., 2003). Total genomic DNA was extracted from each sample using the QIAamp DNA Stool Kit (Qiagen, Crawley, UK) and eluted in 50 µl (Morgan et al., 2009). Each eluate was precipitated (1 µl glycogen, 0.1 vol. 3M sodium acetate pH 5.2, 2 vols ice cold absolute ethanol) as described elsewhere (Sambrook and Russell, 2001) and re-suspended in 5 µl sterile milli-Q water. All four series were supplemented by no-template negative controls (i.e. no DNA in Series A, no oocysts in Series B-D).

2.5.2. Genomic DNA extraction from cloacal swabs
Each cloacal swab was removed from storage at -20 ^{o}C and warmed to room temperature for ~30 min prior to total genomic DNA extraction based upon test protocol D (as described above with the following modifications). One ml milli-Q water, previously warmed to 41 ^{o}C, was pipetted into the plastic cover containing the cloacal swab. After soaking for ≥ 5 min the swab was vortexed and all liquid was collected into a 15 ml disposable polypropylene tube. This wash step was repeated twice, or until no more visible faecal sample could be recovered, pooling the collected faecal homogenate. The faecal homogenate was centrifuged (8 min, 530 x g) and the supernatant was discarded after checking microscopically for oocysts. The pellet was re-suspended in 0.5 ml milli-Q water and transferred to a sterile 2 ml screw-top tube. The 15 ml tube was rinsed with 0.5 ml milli-Q water, which was added to the 2 ml tube. The recovered faecal homogenate was pelleted (1 min, 10,000 x g) and the supernatant was discarded. Total genomic DNA was extracted from the faecal/oocyst pellet as described above except that the final purified DNA pellet was re-suspended in 15 µl sterile milli-Q water (used for triplicate qPCR quantification).

2.5.3. Real-time quantitative PCR
Real-time PCR was performed using the 7500 FAST Real-Time PCR System (Applied Biosystems) with the associated Taqman Fast Universal PCR Master Mix and an *E. acervulina*-specific primer/probe combination as described previously (Eac_qPCRfor: CTCGCGTGTCAGCACTACAT, Eac_qPCRrev: GATACGCTGCTTTGCCTTTC, Eac_qPCRprobe: Fam-TAAGCAACGGGCTACAACCTCACCGTAT-Black Hole Quencher 1; Eurogentec, UK; Blake et al., 2008). Briefly, each sample was amplified in triplicate, based upon a reaction containing 5 µl DNA sample (one replicate sample from Series A-D or one third swab-derived sample), 500 nM forward and reverse primers and 250 nM probe. The Fast qPCR conditions, modified from those suggested by the manufacturer, were 95 ^{o}C for 20 s followed by 40 cycles of 95 ^{o}C for 15 s and 60 ^{o}C for 30 s. Data were collected at the end of each cycle. Triplicate no-template controls were included on every plate in addition to Series A-D no template samples. Additional Series A aliquots were included on every test plate for comparative quantification.

2.5.4. Data analysis
Triplicate cycle threshold data (Ct, the cycle number at which the fluorescence generated through probe cleavage exceeded the no-template control) arising from qPCR of each DNA Series dilution or test sample were averaged and the associated standard error of the mean calculated. Calculations of genome numbers per swab were derived by comparison with Series A, taking account of sample division and dilution during the purification process including an adjustment to account for efficiency of the protocol (derived from comparison between test Series A and D, described below). The efficiency of PCR amplification was determined as described elsewhere using data generated from serial dilution Series A-D Ct values (Cummings and Tarleton, 2003). OPG was calculated for each swab sample by comparison with serial dilution Series D, adjusted according to swab sample weight.

2.6. Statistical comparison of oocyst quantification methods
OPG figures were obtained from 24 h faecal collections (n = 299), single droppings (n = 186) and cloacal swab qPCR (n = 95) for Groups 2-5 and were $\log_{10}$-transformed ($\log_{10}$(OPG+1)). The mean of these $\log_{10}$-transformed counts was used to obtain mean oocyst excretion patterns of each group per day of the experiment.

A subset of these samples (n = 93) was used for the statistical analyses. Single dropping or 24 h faecal collection samples with undetectable levels of oocysts were omitted to obtain normally distributed data. As only two counts above zero remained for the 50 oocyst group, this dose group was omitted completely, leaving 93 observations for the analysis.

Linear mixed effect models with maximum likelihood estimation were carried out using R software (R Foundation for Statistical Computing, 2008), with 24 h faecal collection counts as a dependent variable and single dropping counts as an explanatory variable (model 1), 24 h counts as dependent and qPCR as explanatory variable (model 2) and single dropping counts as dependent and qPCR as explanatory variable (model 3). To correct for repeated measurements in time, "bird" was taken as random effect and "day within bird" was included as random slope. Different variables were tested as explanatory variables in the models, including inoculation dose (500, 50,000 and trickle infection, with 500 as reference group), day, weight of faeces on cloacal swab (models 2 and 3) and several interactions between the explanatory variables. The Akaike's Information Criterion (AIC) was used for model selection and model assumptions were evaluated by examining normality and homoscedascity of the residuals. Bonferroni corrections for multiple comparisons were applied.

3. Results

3.1. Oocyst excretion
All birds inoculated with *E. acervulina* (Groups 2-5) excreted oocysts during these studies except for three birds in the 50 oocyst group (Group 2). No bird in the uninfected control

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group (Group 1) excreted detectable levels of oocysts at any time. Increasing the size of the oocyst dose resulted in elevated levels of oocyst excretion (Fig. 1). Trickle infection resulted in an extended patent period compared to single infection with any dose level (Fig. 1).
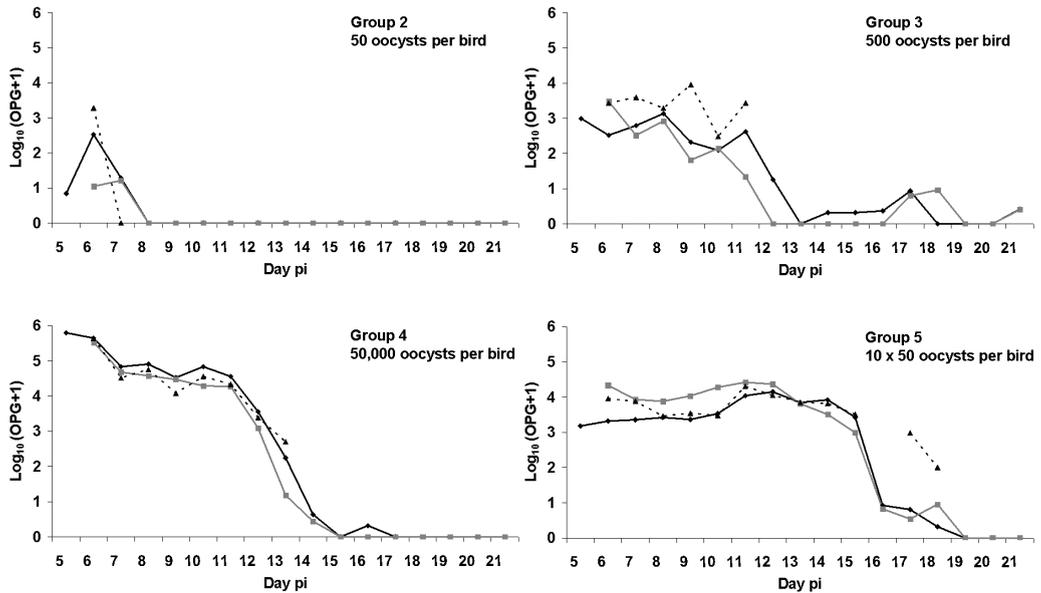
Fig. 1. Mean $\log_{10}$ *E. acervulina* OPG counted microscopically from 24 h faecal collections (—♦—) and single droppings (—■—) and calculated from cloacal swabs by qPCR (--▲--) from days 5 to 21 post-inoculation (D11-D27 of the experiment). For calculation of the mean for Group 2, three birds were discarded, since these remained uninfected. No oocysts were detected in Group 1 (uninfected control).

3.2. Quantification of infection: McMaster oocyst counts from faecal samples

McMaster oocyst counts were carried out on 299 samples from 24 h faecal collections, (representing 24 individual chickens, sampled on 7-16 separate days) and on 186 single droppings (representing 24 individual chickens, sampled on 3-13 separate days). The mean $\log_{10}$-transformed OPG per day was calculated from these samples for each group (Fig. 1). Single dropping oocyst counts were highly comparable to 24 h faecal collection counts during the entire course of the experiment for the different inoculation dose groups (Fig. 1). Data from a selection of 93 samples (as described above) was used for the statistical analysis. Oocyst counts from single droppings were significantly associated with counts from 24 h faecal collections for the different inoculation dose groups (Fig. 2A). The best fitting model describing this association included the single dropping counts and inoculation dose as explanatory variables.
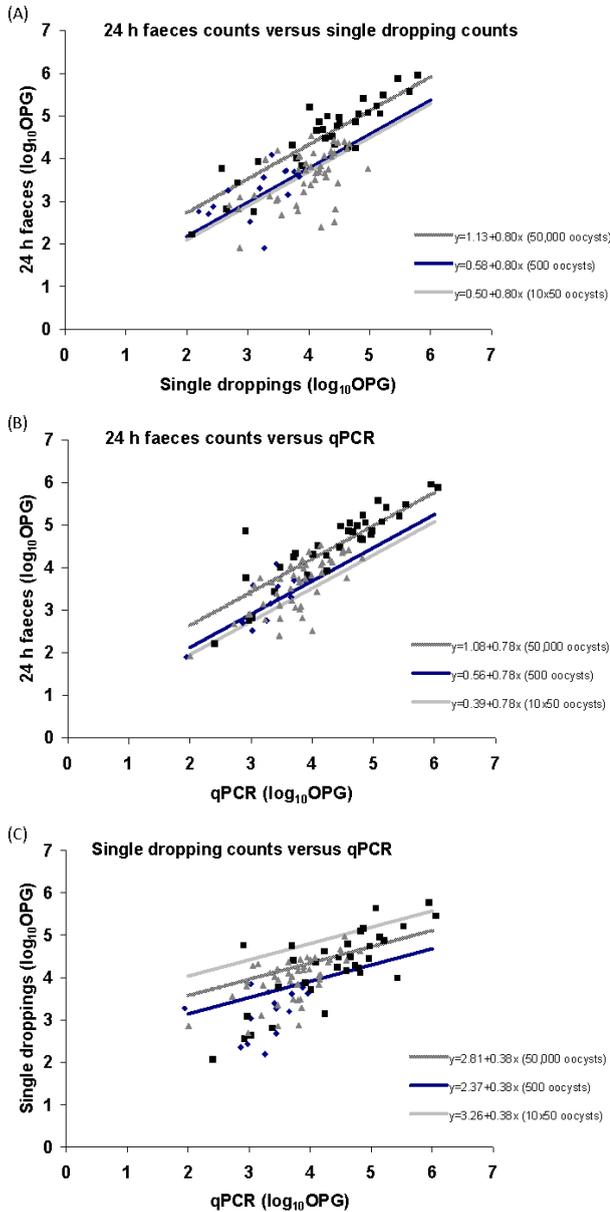
Fig. 2. Linear relationships between oocyst quantification methods. Each graph contains the scatter plot of the data and the corresponding regression line per inoculation dose group (♦ = 500 oocysts per bird, ■ = 50,000 oocysts per bird and ▲ = 10 x 50 oocysts per bird) for $\log_{10}$ OPG from 24 h faeces versus $\log_{10}$ OPG from single droppings (A), $\log_{10}$ OPG from 24 h faeces versus qPCR quantification results (B) and $\log_{10}$ OPG from single droppings versus qPCR quantification results (C). In graph (C) the scatter plot is given for all values, whereas the regression line is only given for day 6 post-inoculation. The intercepts of the regression lines for the other days generally decreased as day post-inoculation increased (not shown in graph).

3.3. Quantification of infection: cloacal swab qPCR

3.3.1. Protocol optimisation

Using purified *E. acervulina* genomic DNA the qPCR assay was found to reproducibly detect one oocyst equivalent (6.2 genomes in this example) but not 0.1 oocyst equivalent (Series A, Table 1). Extracting genomic DNA from purified serial diluted oocysts for qPCR raised the limit of detection by one log dilution (10 oocysts detected but not one, Series B). The presence of faecal material did not affect the limit of detection (Series D) although the addition of a sodium hypochlorite cleaning step limited detection by one further log dilution (Series C). Comparison of Series A and D revealed a reproducible ~30-fold difference between each pure DNA sample and the equivalent number of oocysts after DNA extraction. The coefficient of regression for each test series across the relevant linear range was found to be in excess of 0.93 for all series (Table 1). Purified genomic DNA gave the best regression (Series A), followed by non-sodium hypochlorite treated oocysts in faecal material (Series D).

Table 1

Comparison of *E. acervulina* genomic DNA recovery protocols and subsequent qPCR efficiency. Data derived from 10-fold serial dilution series representing pure *E. acervulina* genomic DNA (A) and total genomic DNA extracted from purified *E. acervulina* oocysts (B; smashed and purified using the Qiagen QIAamp Stool Kit), *E. acervulina* oocysts spiked onto 100 mg chicken faeces (C; sodium hypochlorite treated and then extracted as described for B) and *E. acervulina* oocysts spiked onto 100 mg chicken faeces with no sodium hypochlorite step (D; otherwise as described for C). Each dilution series was based upon a $\log_{10}$ scale of sporulated parasite oocysts (Series B-D) or genomic DNA oocyst equivalents (Series A, assuming 6.2 genomes per oocyst on average from an ~70% sporulated oocyst sample).

Series	Template	Number of oocysts[a]		Coefficient of regression[b]	Efficiency of PCR[c]
		Range	Linear range		
A	gDNA	10^6 to 10^{-1}	10^6 to 10^0	0.9959	1.89
B	Oocysts	10^6 to 10^0	10^6 to 10^1	0.9518	1.99
C	Oocysts	10^6 to 10^0	10^6 to 10^2	0.9316	1.94
D	Oocysts	10^6 to 10^0	10^6 to 10^1	0.9908	1.92

[a] The range of oocysts per dilution or, where purified genomic DNA was used, the number of "oocyst equivalents" based upon the comparative oocyst sporulation rate.
[b] The coefficient of regression was measured across the linear range.
[c] Efficiency of PCR based upon Ct values, not 40-Ct values.

3.3.2. Cloacal swab qPCR

In total 95 cloacal swabs were selected for qPCR analysis, representing 19 individual chickens sampled on 1-9 separate days. All 95 swabs were derived from birds shown by 24 h faecal collection and single dropping counts to be excreting *E. acervulina* oocysts. Swab sample weight and the related 24 h faecal collection oocyst count were used to estimate the likely oocyst load per swab (based upon the unlikely assumption of equal oocyst shedding). Thus, a minimum of ~2.5 and a maximum of 1.64×10^5 oocysts were predicted per swab. Parasite quantification by qPCR identified *E. acervulina* genomic DNA from 94 of the 95 swabs tested, with only the swab predicted to carry the lowest oocyst load negative. Cloacal swab parasite

quantification was directly comparable to 24 h faecal collection and single dropping counts (Fig. 1). Data from 93 swabs were included in the statistical analysis (as described above). Cloacal swab qPCR quantifications were significantly associated with oocyst counts from 24 h faecal collections (Fig. 2B) and with single dropping oocyst counts (Fig. 2C) for the different inoculation dose groups. The best fitting model for the association between cloacal swab qPCR quantifications and 24 h faecal collection counts included qPCR results and inoculation dose as explanatory variables. For the association between cloacal swab qPCR quantifications and single dropping counts, qPCR results, inoculation dose and day were significant explanatory variables in the best fitting model.

4. Discussion

The objective of this study was to determine whether two "bird-specific" oocyst quantification techniques, i.e. oocyst counts from single droppings and qPCR quantification of oocysts on cloacal swabs, are good and practically applicable measures to quantify eimerian infection in individual chickens. Performance of the different techniques was tested for a variety of oocyst numbers per sample, that were obtained by using low, medium and high inoculation doses and through sampling at start and end of the patent period. This study showed that both single dropping counts and qPCR results were significantly associated with 24 h faecal collection counts across the spectrum of samples. Furthermore, oocyst excretion patterns based on single dropping counts and cloacal swab qPCR results were highly comparable with each other and to 24 h faecal counts during the entire course of the infection for all groups, suggesting that both protocols reliably quantify oocyst output. Both sampling methods are easily applicable to individuals in a flock, most notably cloacal swabbing for qPCR.

   Comparison of oocyst counts from single droppings by microscopy and cloacal swabs by qPCR with 24 h faecal collection counts yielded slightly higher estimations of OPG, in contrast with the slightly lower estimation of OPG using qPCR on faecal samples described by Morgan et al. (2009). An explanation for our findings might be a higher degree of oocyst degradation in 24 h faeces (Williams, 1995a), where oocysts are exposed to adverse conditions in the faeces for a slightly longer period compared to single droppings or cloacal swabs. In addition, the higher OPG estimated by qPCR could have been caused by the presence of *Eimeria* lifecycle stages that were not identified using a microscope but persisted within the sample in the absence of a sodium hypochlorite step, e.g. merozoites (Morgan et al., 2009), although the labile nature of these lifecycle stages makes it unlikely that they would have survived the freezing and sample recovery procedures. Alternatively, the lack of sodium hypochlorite treatment may have supported the retention of a larger quantity of non-parasite nucleic acid (i.e. derived from the host or feed), which could have acted as a carrier during DNA purification and enhanced sensitivity. Furthermore, although fresh faecal samples are

expected to contain mainly unsporulated oocysts, a small proportion of sporulated oocysts ingested from the litter can pass through the gut and can be recovered intact from the faeces, as demonstrated by Williams (1995b). Possibly, OPG estimated by qPCR was slightly higher due to the presence of small numbers of these sporulated oocysts, each of which contains four times as much DNA as unsporulated oocysts. An increased load of sporulated oocysts in the litter as the experiment progressed might explain why the higher estimation of OPG by qPCR on cloacal swabs compared to single dropping counts increased throughout the course of the experiment.

In this study all cloacal swab samples were stored frozen before qPCR quantification, so all oocysts were assumed to be unsporulated, possibly with the exception of a small number of sporulated oocysts passing through the gut, as discussed above. When using this qPCR with field samples, vaccines or stored samples containing sporulated oocysts, representing four times as much nuclear DNA as unsporulated oocysts, a correction for sporulation percentage such as that suggested by Morgan et al. (2009) could be used. The use of freshly excreted single droppings or cloacal swabs indicates that most, if not all oocysts will remain unsporulated, an advantage that removes the uncertainty of sporulation rate when using genome quantification as a measure of parasite burden.

Performance of qPCR compared with oocyst counts was assessed for *E. acervulina* in this study. It is expected that single dropping counts for *E. tenella* might be difficult to collect due to the intermittent excretion of caecal droppings and that cloacal swabs, likely to contain mainly colonic faeces, may not accurately represent caecal *E. tenella* infection. Such problems are not anticipated to be as significant for the other *Eimeria* species that are excreted with colonic faeces, although further experiments should be carried out to compare performance of the three enumeration strategies with different *Eimeria* species and isolates.

Compared to bulked 24 h faecal samples, single dropping counts and qPCR have the advantage that oocyst excretion in individual birds at time of sampling can be assessed, providing an important diagnostic tool for determining infection dynamics. Both techniques are relatively labor-intensive, although efficiency increases rapidly using qPCR when sample size increases, whereas microscopic counting of single droppings remains time-consuming despite the number of examined samples. Furthermore, qPCR can be carried out on samples that have been stored for extended periods, e.g. in a freezer, allowing for large sample sizes in large scale experiments and epidemiological studies in the field. An additional advantage of qPCR is the ability to differentiate between different *Eimeria* species using multiple assays or a multiplex strategy. The extension of these studies to use qPCR with single droppings can decrease the duration of laboratory manipulation required and may be expected to yield further protocol improvements.

Acknowledgements

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Chapter 3

Eimeria acervulina: The influence of inoculation dose on transmission between broiler chickens

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Abstract - The course and clinical appearance of an *Eimeria* species infection in chicken flocks depend on the response of an individual bird to infection and on population-dynamics of the infection in the flock. Differences in ingested numbers of oocysts may affect oocyst load in the flock and the subsequent infectious dose for not yet infected birds. To study the link between numbers of oocysts excreted by infected birds and transmission of *Eimeria acervulina*, experiments were carried out with 42 pairs of broiler chickens using inoculation doses with 5, 50, 500 or 50,000 sporulated oocysts. In each pair one bird was inoculated and the other bird was contact-exposed. All contact birds became infected, which occurred on average within 34 hours after exposure to an inoculated bird. Although a higher inoculation dose resulted in higher oocyst excretion in inoculated and contact-infected birds, only small non-significant differences in transmission rates between groups were found.

Keywords - *Eimeria acervulina;* protozoa; transmission rate; broilers; population-dynamics

1. Introduction

Coccidiosis, caused by protozoan parasites of the genus *Eimeria*, is a common disease of poultry, causing considerable economic losses in the poultry industry (Williams, 1999). Losses are attributed to costs of medication for prevention and treatment and to mortality, malabsorption, inefficient feed utilisation and impaired growth rate of chickens (Williams, 1999, Dalloul and Lillehoj, 2005). Increasing resistance of the *Eimeria* species against coccidiostatic drugs (Chapman, 1997, Mathis and Broussard, 2006, Peek and Landman, 2006), regulations and bans on the use of anticoccidial drugs e.g. the proposed phasing out of coccidiostatic drugs in the EU by 31 December 2012 (European Community, 2003), and lack of success in producing cost-effective vaccines require the development and evaluation of alternative intervention strategies to improve the control of *Eimeria* species infections in the future.

The efficacy of control measures is often studied in challenge experiments in which immunological responses, oocyst output, and the birds performance after inoculatíon with a fixed dose of *Eimeria* oocysts was determined (Danforth et al., 1997, Dalloul et al., 2002, Talebi and Mulcahy, 2005). One of the factors that affect the response of an individual bird to an infection with *Eimeria* species is the number of ingested oocysts (Lillehoj, 1988, Conway et al., 1993, Graat et al., 1996, Blake et al., 2005). It is known, for instance, that increasing doses of oocysts generally give rise to progressively higher oocyst yields in inoculated birds, until a certain infectious dose is reached, the "crowding threshold" (Williams, 2001). In challenge experiments the inoculation dose is known rather precisely, as birds are artificially infected, but the infectious dose ingested by birds in a flock is unknown. Moreover, although an infection can be established after a single uptake of a small number of oocysts (Norton and Joyner, 1986), the outcome of the infection (e.g. clinical signs, infectivity) not only depends on oocysts dose alone, but may also depend on the distribution of the oocyst uptake over time (Joyner and Norton, 1976, Galmes et al., 1991, Stiff and Bafundo, 1993). Finally, as the number of oocysts in the environment may change over time during the spread of the infection in the flock, the ingested dose for naturally exposed birds in a flock may change in time as well.

This implies that the mutual interaction between the responses to infection in individual birds and between infectious and susceptible birds in a flock and their environment will affect the dynamics of the infection in a flock. Although this seems straightforward, the outcome of these interactions may give rise to non-linear effects, which may result in a counterintuitive and an unpredictable course of the infection (Roberts and Heesterbeek, 1995, 1998; Graat et al., 1996, Klinkenberg and Heesterbeek, 2007). Consequently, when studying the course of infection or the efficacy of control measures on flock level, aspects regarding population-dynamics should be determined as well.

Dynamical aspects of the infection, however, cannot be studied in challenge experiments, as all birds are artificially inoculated and at the same time. A transmission experiment, in

which transmission from infected to contact-exposed birds can be quantified, is more appropriate to study population-dynamics (Velthuis et al., 2007).

To determine the effect of ingested dose on oocyst output and transmission rates, i.e. the probability of infection per unit of time, we carried out experiments with 42 pairs of broiler chickens using four inoculation doses. In each pair one bird was inoculated with *Eimeria acervulina*. Oocyst output was determined for each bird, and the transmission rate was calculated based on the number of contact-infected birds for each dose group. This approach was considered to be appropriate, because the amount of oocysts shed in the litter can be quantified adequately and it can be subsequently determined to which oocyst level the contact bird has been exposed. These experiments may be a first step to unravel the interactions between the infected and contact bird, and may give more insight in the effect of ingested dose on infection dynamics of *Eimeria* species between birds, which can be helpful to further study the population-dynamics of *Eimeria* species in poultry flocks.

2. Materials and methods

2.1. Chickens and management
Four experiments were carried out with male broiler chicks purchased at day of hatch (day 0) from a specified pathogen free (SPF) parent flock, a crossbred of Cobb, Hybro and Ross (GD-Animal Health Service (AHS), Deventer, the Netherlands). SPF chickens were used to prevent the influence of other factors than inoculation dose on transmission, i.e. presence of maternal antibodies or other infectious agents affecting the intestinal tract. This parent stock was tested regularly for absence of coccidia and other pathogens. On arrival at the experimental facilities, all birds were tagged for identification. Chicks were housed in pairs: in experiment I negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, the Netherlands) were used, each containing one pen for a single pair of birds, and in experiments II-IV pairs were housed in floor pens. All pens contained one kg of wood shavings per m^2. The surface of the pens in experiments II and III was 0.1 m^2. For experiments I and IV larger pens were used (0.3 m^2) to make a longer duration of the experiment possible. Experiments II and III were carried out simultaneously in a single experimental unit. Experiments I and IV were carried out separately at a different facility.

The temperature of the environment was gradually decreased from 34 °C at day 0 to approximately 19 °C from day 35 onwards. The lighting scheme was 23 hours of light per day. Tap water and a broiler starter ration (2900 kcal/kg of metabolisable energy and 21.6% crude protein), without anticoccidial drugs were provided *ad libitum*.

The birds were observed twice daily for signs of disease or welfare impairment. The experiments were approved by the Animal Experimental Committees of Wageningen University and Utrecht University, in accordance with the Dutch law on experimental animals.

2.2. Experimental design

Four experiments were carried out with inoculation doses of 5, 50, 500 or 50,000 sporulated oocysts of *E. acervulina* (experiments I-IV, respectively). At day of inoculation (referred to as day 0 post-inoculation, "pi"), the age of birds was 2 (experiment I), 6 (experiments II and III) or 24 days (experiment IV). The inoculum was administered to one bird per pair (the inoculated bird, referred to as *I* bird). The non-inoculated pen mate was contact-exposed (the contact bird, referred to as *C* bird) from day 1 pi onwards. The number of pairs was 9, 17, 10 and 6 for experiments I-IV respectively. In experiments II-IV, non-inoculated sentinel birds (10 for experiments II and III and 2 for experiment IV) were randomly placed in floor pens between the cages to detect possible between-cage transmission. The birds were housed together for at least 26 days.

2.3. Inocula and inoculation

We used sporulated *E. acervulina* oocysts (Weybridge W119, supplied by AHS; originally provided by the Central Veterinary Laboratory in Weybridge, United Kingdom), stored at 8 °C in 2.5% (w/v) potassium dichromate.

For experiment I oocysts, suspended in PBS-BSA solution, were placed in a Terasaki plate well (Microwell® plate, Nunc™ T-3017-2, Denmark) containing 4 μl of PBS-BSA, using a pasteur pipette under 50 x magnification with a binocular microscope. After counting and determination of sporulation, using 10 x 40 magnification with a stereo microscope, the content of the wells with 5 sporulated oocysts was brought into a gelatinous capsule (volume 0.20 ml). Twenty μl of PBS-BSA was added to the capsule to prevent desiccation of oocysts. The capsule was placed in the broiler's oesophagus using a 1 ml syringe, followed by some tap water.

For experiments II-IV a stock suspension of sporulated oocysts was diluted to obtain suspensions with 50 (experiment II) or 500 (experiment III) sporulated oocysts per 0.9 ml tap water or 50,000 sporulated oocysts per 1 ml (experiment IV). A Fuchs-Rosenthal haemocytometer counting chamber was used to determine number of sporulated oocysts. The suspension was orally inoculated directly into the crop using a 1 ml syringe.

2.4. Sampling of faecal droppings

Faecal droppings were collected before the start of the experiment to verify absence of *Eimeria* species using the sedimentation-flotation technique (Long et al., 1976). Single individual droppings were collected daily from day 3 pi onwards as follows. Each chick was placed in a box with a clean paper sheet for one to two hours. The droppings were collected and stored at 4 °C until further processing described below.

To quantify the number of oocysts per gram of faeces (OPG), we used a modification of a McMaster oocyst counting chamber technique (Long and Rowell, 1958). Briefly: the single dropping was homogenised in 20 ml salt solution (151 g/L). Then, 2 ml was added to 8 ml of a saturated salt solution (311 g/L), and this suspension was used to fill two McMaster counting

chambers (2 x 0.15 ml). OPG was calculated: 333.3 x total number of oocysts x dilution factor (1 if no further dilution, 10 for 10-fold dilution)/sample weight.

If no oocysts were found, a modification of the sedimentation-flotation technique was applied using 1 g of faecal material (Long et al., 1976), that was taken from the single dropping prior to applying the McMaster technique.

2.5. Data analysis

2.5.1. Oocyst output data

Oocyst counts were $\log_{10}$-transformed to normalise the data ($\log_{10}$ (OPG+1)). The mean of the $\log_{10}$-transformed OPG was calculated for *I* and *C* birds per group per day to obtain mean oocyst excretion patterns. As a measure for the total number of excreted oocysts, the *AUC* (area under the curve) of oocyst output was calculated per bird from the daily OPG results and was $\log_{10}$-transformed (referred to as $\log_{10}$ *AUC*). The $\log_{10}$ *AUC* was calculated per bird for the following periods of the experiment to facilitate comparisons between *I* and *C* birds and dose groups: total experiment (days 4-30 pi); first excretion peak of *I* birds (days 4-8 pi); second peak of *I* birds or first excretion peak of *C* birds (days 9-13 pi); and a period in which both *I* and *C* birds are comparable regarding exposure to infectious oocysts in the litter and are all actively shedding (days 9-24 pi). For *I* and *C* birds of each group, the mean of day at start of oocyst excretion and the mean of the $\log_{10}$ *AUC* for the different periods was calculated. The 95% confidence intervals (CI) were calculated for start of excretion and $\log_{10}$ *AUC* data, based on the normal distribution.

If results according to the sedimentation-flotation test were negative, McMaster counts were considered to be zero for the calculation of the mean $\log_{10}$ OPG and $\log_{10}$ *AUC*. In a few cases, primarily at the beginning and end of oocyst excretion when oocyst output was low (Tables 1-4), McMaster counts were negative whereas sedimentation-flotation results were positive. For these cases quantitative information on oocyst output was not available and these cases were considered as missing values for calculation of the mean $\log_{10}$ OPG and $\log_{10}$ *AUC*. Statistical analyses were carried out using SAS for Windows 9.1 (SAS, 2003). Linear regression models were used with variables *first day of excretion I bird, first day of excretion C bird* and $\log_{10}$ *AUC* for different periods as dependent variables and *inoculation dose* (5, 50, 500 or 50,000) and *bird type* (*I*, *C*) as explanatory variables. If relevant, $\log_{10}$ *AUC* variables and interaction terms were added to the model as explanatory variables. In models where $\log_{10}$ *AUC* data was analysed from day 9 pi onwards, a linear mixed model was used with *cage* as random factor, to correct for mutual influence on oocyst output of *I* and *C* birds in the same cage. The two-tailed partial F-test (type III) was used as the elimination criterion for the variables and fit of the model was assessed by the Akaike's Information Criterion for linear mixed models and R^2 for general linear models. Bonferroni corrected pair-wise multiple *t*-tests were carried out to test the difference between each pair of means. Model assumptions were evaluated by examining normality and equality of variances of the

residuals. A two sample paired *t*-test was carried out to compare the first and second excretion peak in *I* birds. *P* values < 0.05 were considered significant.

2.5.2. Transmission rate calculations

A stochastic susceptible-infectious (SI) model was used to describe the transmission of *E. acervulina* from "infectious" (inoculated birds, *I* birds) to "susceptible" (contact-exposed birds, *C* birds). The SI model assumes that when a susceptible chicken becomes infected, in this case through contact with oocysts in the litter excreted by an infectious bird, they remain infectious for the duration of the experimental period (Bailey, 1975), since it is known that sporulated oocysts can remain infectious in litter for several weeks (Williams, 1995a).

The rate of new infections is defined as βSI/*N* (*N* is the total number of birds in the group). Transmission rate parameter β is defined as the number of new infections that occurs due to (indirect) contact with one infectious animal per unit of time in a large, completely susceptible population (Anderson and May, 1991). This definition suggests that the force of infection increases with the prevalence of infection *I/N*, i.e. "frequency-dependent transmission" (de Jong et al., 1995). By another frequently used assumption the force of infection increases with the number of infectious birds I, i.e. "density-dependent transmission". In the current context, where only pairs of birds are considered, the choice of methods is only a matter of scaling and will not affect any conclusion on differences between dose groups.

Two states can be distinguished: the initial state with one infectious bird and one contact bird and the state in which the susceptible contact-exposed bird has become infectious. Because we do not observe the continuous process, we rewrite this into a probability per day p that the contact bird gets infected, if it is at risk:

$$p = 1 - \exp(-\beta SI/N) = 1 - \exp(-\beta/2)$$

The probability p is estimated as:

$$\hat{p} = \text{(number of contact birds infected)/(number of days at risk)}$$

The number of days at risk is the sum of the number of days at risk with a contact-infection (the number of contact birds infected) and the number of days at risk without contact-infection, which we call the *total infection lag time*. It is the sum of *infection lag times* of single pairs, defined as the number of days that the contact bird was at risk but not infected (Tables 1-4 and Appendix A). This probability of infection, p , can be used to estimate the transmission rate per day, β, using the following equation:

$$\overline{\beta} = -2\ln(1 - \widehat{p})$$

In Appendix A the estimation procedure is explained in more detail, including the derivation of confidence intervals, and the hypothesis test to test differences between groups.

3. Results

Inoculation was successful in all cases, and all contact birds got infected. In samples of non-inoculated sentinel chickens, small numbers of oocysts were detected from day 12 pi (experiments II and III) and day 14 pi (experiment IV) onwards, which was after all contact birds had become positive. Therefore the transmission events in each pen are reasonably assumed to be independent.

Oocyst count data, number of infected contact birds and *infection lag times* are summarised for each experiment in Tables 1-4. Inoculated birds excreted oocysts from, on average, day 5 pi onwards. Linear regression analyses showed that inoculated birds in the 50,000 oocyst group started oocyst excretion significantly earlier than those in the 5 (P = 0.013) and 50 oocyst groups (P = 0.002). The difference with inoculated birds of the 500 oocyst group was not significant (P = 0.333) (Table 5). All contact birds became infected, and shed oocysts from, on average, day 10 pi onwards. Contact birds in the 5 oocyst group started oocyst excretion significantly later than contact birds of the 50,000 (P = 0.044) and 500 oocyst group (P = 0.047) (Table 5), independent on the start of excretion by their inoculated pen mate.

Mean oocyst excretion patterns are shown in Fig. 1A-D for all groups. Oocyst output data during different periods of the experiment are given in Table 5. Oocyst output of inoculated birds during their first excretion peak (days 4-8 pi) differed significantly between all dose groups (P values ranging from < 0.0001 to 0.033). Birds in the 50,000 group had the highest output, followed by the 500 and the 5 oocyst group. The 50 oocyst group had the lowest initial oocyst output (Table 5). A second oocyst excretion peak, that was significantly higher than the first excretion peak and coincided with the start of shedding of the contact birds, was observed for inoculated birds of the 5 (t = -2.73, Prob > t = 0.026), 50 (t = -5.14, Prob > t = 0.0001), and 500 oocyst group (t = -3.65, Prob > t = 0.007) (Fig. 1A-C). A detectable second excretion peak was not found for inoculated birds in the 50,000 oocyst group (Fig. 1D). Total output in both inoculated and contact birds (from days 4-30 pi) was highest for the 50,000 oocyst group, followed by the 500, 5 and 50 oocyst group.
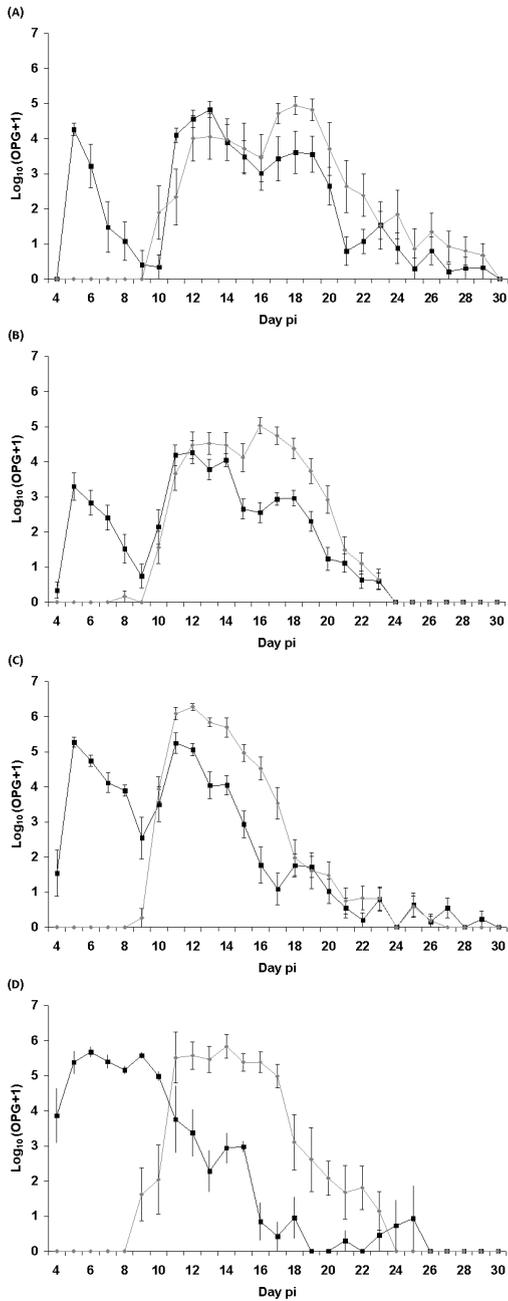
Fig. 1. Mean oocyst production in $\log_{10}$ (OPG+1) for the inoculated (—■—) and contact birds (—♦—) of the 5 oocyst group (A), 50 oocyst group (B), 500 oocyst group (C) and 50,000 oocyst group (D). In cases of missing values due to absence of single droppings, the mean was calculated using the available oocyst counts.

Table 1

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique for the 5 oocyst group of experiment I.

Bird[a]	Day[b] 5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	Lag[c] 1	2
	Day pi 3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	1	2
5I	**	*-	+*	++	--	*-	++	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	-*	--	++	+*	-*	-*	-*	-+	-+	-+	5	
1C	**	*-	*-	--	*-	*-	*-	*-	--	*-	++	++	*-	-+	++	+*	+*	+*	+*	+*	+*	+*	-*	+*	-+	--	-+		
26I	**	*-	+*	+*	+*	-+	*-	*-	++	+*	+*	+*	+*	+*	-*	+*	+*	-*	--	-+	-*	+*	-*	+*	-+	-+	+-	2	
19C	**	*-	*-	*-	*-	--	*-	++	--	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	-+	+*	-+	--	-*	**	-+	-+		
23I	**	*-	+*	+*	--	*-	*-	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	++	+*	-*	+*	-*	-*	-+	-+	4	
4C	**	*-	*-	*-	*-	*-	*-	*-	--	++	*-	--	*-	-+	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-+		
6I	**	*-	+*	--	--	++	*-	--	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	-+	+*	-*	**	**	--	--	-+	2	
9C	**	*-	*-	*-	*-	--	*-	++	+*	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	+*	+*	+*	++	-+		
20I	**	*-	++	+*	-+	++	*-	*+	++	+*	+*	--	*-	-+	++	+*	+*	+*	--	++	+*	+*	-*	-*	-+	-+	--	2	
16C	**	*-	*-	-+	*-	*-	*-	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	--	+*	-*	-*	-*	++	++	++		
31I	**	*-	+*	--	*+	+-	*-	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-+	+*	-*	-*	-+	-+	-+	-+	3	
8C	**	*-	*-	*-	*-	--	*-	--	+-	+*	+*	++	+*	+*	+*	+*	+*	+*	+*	-+	-*	-*	-*	-*	-*	--	--		
21I	**	*-	+*	+*	-*	--	*-	*-	++	+*	+*	+*	+*	+*	+*	++	*-	-+	++	+*	-+	-+	*+	-+	-*	-+	--	4	
30C	**	*-	*-	*-	*-	*-	*-	--	--	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	-*	-+	+-		
24I	**	*-	++	+*	+*	-*	*-	++	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	--	+*	+*	+*	-*	+*	--	++	--	2	
25C	**	*-	*-	*-	*-	*-	*-	+*	+*	++	+*	+*	+*	+*	+*	+*	+*	-*	--	+*	-*	-*	-*	-*	-+	-+	++		
27I	**	*-	+*	+*	++	*-	*-	*-	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-+	+*	-+	-*	-*	+*	+-	**	**	3	
2C	**	*-	*-	*-	*-	*-	*-	--	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	--	+*	-*	+*	-*	**	**	**	**		
Total infection lag time[d]																												27	
Total number of infected contact birds																												9	

Oocyst output data following each *I* and *C* bird: the first symbol represents the results of McMaster oocyst counts, the second symbol shows the result of sedimentation-flotation slides: +: oocysts were detected, *: not done, -: no oocysts were detected. [a-d] See footnotes for Table 4.

Table 2

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique for the 50 oocyst group of experiment II.

Bird[a]	Day[b] 5	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	Lag[c] 1	2
	Day pi -1	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1	2
518I	*_	_*	+*	+*	+*	+*	_*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	*_	*_	**	--	*_	*_	**	--	**	3	3
514C	*_	**	**	**	*_	--	*_	--	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	**	--	*_	*_	**	--		
523I	*_	_*	**	+*	+*	_*	_*	_*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-+	*_	**	--	*_	*_	**	--	**	2	2
540C	*_	**	**	**	**	_*	*_	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	*_	**	--	*_	--	**	--	**		
512I	*_	_*	**	+*	+*	_*	+*	+*	+*	+*	+*	+*	+*	_*	+*	+*	+*	_*	+*	-+	*_	**	-+	--	*_	**	--	**	3	3
559C	*_	**	**	**	**	--	*_	--	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	**	--	*_	*_	**	--		
526I	*_	_*	**	+*	_*	_*	+*	+*	+*	+*	+*	**	+*	+*	+*	+*	+*	_*	_*	++	-+	**	-+	--	*_	**	--	**	0	0
567C	*_	**	**	**	_*	+*	*_	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	+*	_*	**	_*	**	--	-+	-+	**	--		
547I	*_	_*	+*	+*	+*	_*	_*	_*	+*	+*	+*	**	_*	+*	+*	+*	+*	+*	+*	+*	+*	**	--	-+	--	**	--	**	2	2
508C	*_	**	**	**	**	_*	*_	+-	++	_*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	**	--	*_	*_	**	--		
513I	*_	_*	+*	_*	+*	_*	_*	_*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	**	-+	-+	-+	-+	--	**	3	3
515C	*_	**	**	**	_*	*_	--	++	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	*_	**	--	*_	*_	**	--	**				
507I	*_	_*	+*	_*	+*	_*	_*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	+*	**	--	*_	*_	**	--	4	3
531C	*_	**	**	**	**	_*	*_	--	-+	_*	+*	_*	_*	+*	_*	+*	+*	+*	+*	+*	+*	+*	**	--	*_	*_	-+	*_		
528I	*_	_*	+*	+*	+*	+*	_*	_*	+*	+*	+*	+*	+*	_*	+*	+*	+*	_*	+*	_*	*_	**	--	*_	*_	**	--	**	3	3
601C	*_	**	**	**	**	--	*_	_*	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	*_	**	-+	--	*_	**	--	**		
538I	*_	_*	+*	+*	+*	+*	_*	_*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	-+	**	--	*_	*_	**	--	**	3	3
539C	*_	**	**	**	**	--	*_	--	++	**	+*	+*	+*	+*	+*	**	+*	+*	+*	+*	+*	--	*+	--	*_	--	**			
533I	*_	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	--	*_	*_	**	**	**	**	**	**		2	2
554C	*_	**	**	**	**	_*	*_	++	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	-+	-+	-+	**	--	*_	*_	**	--	**		
529I	*_	_*	+*	+*	+*	+*	_*	_*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	_*	**	-+	--	*_	**	--	**	3	2
542C	*_	**	**	**	**	_*	*_	-+	++	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	_*	**	--	*_	*_	**	--	**			
536I	*_	+*	+*	+*	+*	+*	_*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	**	--	*_	*_	**	--	**		2	2
563C	*_	**	**	**	_*	_*	*_	+-	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	**	-+	--	*_	*_	**	--	**			
557I	*_	_*	+*	+*	+*	_*	_*	+*	+*	+*	+*	+*	+*	+*	_*	_*	+*	_*	_*	-+	**	--	*_	*_	**	--	**		4	4
558C	*_	**	**	**	*_	_*	*_	--	--	+*	+*	**	+*	+*	+*	+*	+*	+*	_*	+*	**	--	*_	*_	**	--	**			
549I	*_	_*	_*	+*	_*	_*	_*	_*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	**	--	*_	*_	**	--	**	2	2
506C	*_	**	**	**	**	_*	*_	+-	+*	+*	_*	_*	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**		
566I	*_	_*	+*	+*	+*	+*	+*	+*	+*	+*	**	+*	+*	+*	+*	_*	_*	++	+*	-+	--	*_	-+	-+	**				2	0
570C	*_	**	**	**	**	-+	*_	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	*_	-+	**	--	*_	*_	**	--	**		
564I	*_	_*	_*	_*	_*	+*	_*	+*	+*	_*	_*	_*	+*	_*	+*	+*	+*	+*	_*	-+	*_	**	--	*_	*_	**	--	**	3	3
551C	*_	**	**	**	**	_*	*_	--	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	+*	_*	**	-+	--	*_	**	--		
532I	*_	_*	+*	+*	+*	_*	_*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	_*	*_	*_	**	-+	--	*_	**	--	**	2	2
516C	*_	**	**	**	**	_*	*_	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	_*	**	--	*_	*_	**	--	**		
Total infection lag time[d]																													43	39
Total number of infected contact birds																													17	

Oocyst output data following each *I* and *C* bird: the first symbol represents the results of McMaster oocyst counts, the second symbol shows the result of sedimentation-flotation slides: +: oocysts were detected, *: not done, -: no oocysts were detected. [a-d] See footnotes for Table 4.

Table 3

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique for the 500 oocyst group of experiment III.

Bird[a]	Day[b] 5	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	Lag[c] 1	Lag[c] 2
	Day pi −1	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1	2
520I	*-	-*	+*	+*	+*	+*	-*	-*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	+*	**	--	*-	*-	**	--	**	2	2
501C	*-	**	**	**	--	--	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	--	++	-*	**	++	-+	-+	-+	**		
552I	*-	-*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	+*	-*	+*	**	++	-+	--	--	**	2	2
556C	*-	**	**	**	**	--	*-	++	+*	+*	+*	+*	+*	**	+*	+*	-*	-*	--	++	-*	-*	**	++	--	-+	-+	**		
541I	*-	-*	+*	+*	+*	+*	-*	+-	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	-*	+*	-*	-*	**	-+	-+	--	**	**	2	2
524C	*-	**	**	**	--	--	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	+*	**	-+	-+	-+	-+	--	**		
568I	*-	-*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	-*	+*	+*	-*	-*	*-	*-	**	--	-+	--	**	--	**		2	2
548C	*-	**	**	**	**	--	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	-*	++	+*	**	++	+-	-+	-+	--	**		
503I	*-	-*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	-*	+*	+*	+*	-*	-*	-+	**	--	-+	++	-+	--	**		2	2
522C	*-	**	**	**	**	--	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	+*	-*	-*	++	**	-+	-+	--	**	**		
505I	*-	-*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	-*	+*	-*	-*	--	*-	*-	**	--	*-	*-	**	--	**	2	0
572C	*-	**	**	**	**	-+	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	-*	-+	**	--	*-	*-	**	--	**		
560I	*-	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	-*	-+	**	++	++	++	-+	--	**	2	2
511C	*-	**	**	**	**	--	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	**	-+	--	*-	**	--	**		
546I	*-	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	-*	-*	+*	+*	+*	-*	+*	**	--	*-	*-	**	--	**	3	3
525C	*-	**	**	**	**	--	*-	**	+*	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**		
504I	*-	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	+*	-*	-*	*-	--	**	--	*-	*-	**	**	1	1
509C	*-	**	**	**	**	--	+*	+-	++	+*	+*	+*	+*	+*	+*	+*	-*	-*	-+	--	++	-*	**	--	*-	*-	**	--		
530I	*-	+*	+*	+*	+*	+*	-*	+*	+*	+*	+*	+*	+*	-*	-*	+*	+*	-*	-*	*-	++	**	++	-+	++	**	++	**	2	2
502C	*-	**	**	**	**	--	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	+*	-*	-*	**	--	*-	--	**	--	**		
Total infection lag time[d]																													20	18
Total number of infected contact birds																													10	

Oocyst output data following each *I* and *C* bird: the first symbol represents the results of McMaster oocyst counts, the second symbol shows the result of sedimentation-flotation slides: +: oocysts were detected, *: not done, -: no oocysts were detected. [a-d] See footnotes for Table 4.

Table 4

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique for the 50,000 oocyst group of experiment IV.

Bird[a]	Day[b]	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	Lag[c]	
	Day pi	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	1	2
827I		+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	++	++	-*	-*	++	--	*_	*_	**	-+	*_	*_	**	1	
803C		**	**	**	**	*_	+-	--	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	--	--	**		
764I		-*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	++	-*	--	-+	*_	-+	*_	**	*_	**	**	**	2	
756C		**	**	**	**	*_	*_	++	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	--	-+	**		
311I		+*	+*	+*	+*	+*	+*	+*	**	+*	-*	++	+*	-*	-+	-+	*_	*_	*_	**	*_	**	**	**	1	
823C		**	**	**	**	--	++	++	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	-*	--	**		
741I		+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	++	-*	-+	++	--	-+	-+	--	*_	*_	**	**	2	
847C		**	**	**	**	--	--	++	+*	+*	+*	+*	+*	+*	+*	+*	-*	-*	--	++	--	--	*_	**		
310I		+*	+*	+*	+*	+*	+*	**	-*	--	+*	+*	+*	+*	-*	--	*_	*_	*_	**	**	**	*_	**	3	
839C		**	**	**	**	--	--	--	++	+*	+*	+*	+*	+*	+*	+*	-*	+*	+*	-*	-+	+-	--	**		
795I		+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	**	-+	--	++	**	++	++	**	1	
857C		**	**	**	**	*_	+-	-+	++	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	-+	++	--	-+	**		
Total infection lag time[d]																										10
Total number of infected contact birds																										6

Oocyst output data following each *I* and *C* bird: the first symbol represents the results of McMaster oocyst counts, the second symbol shows the result of sedimentation-flotation slides: +: oocysts were detected, *: not done, -: no oocysts were detected.

[a] Individual tag number followed by bird type *I* or *C*.

[b] Age of the birds in days. Below: day pi, where day pi = 0 is day of inoculation of *I* birds.

[c] *Infection lag time* = the number of days between the first day when it is considered possible for a contact bird to start excreting oocysts, i.e. at day 8 pi and the first day when the contact bird actually started to excrete oocysts, according to McMaster oocyst counts (*Lag 1*) or adjusted for sedimentation-flotation results (*Lag 2*: when birds were positive according to the sedimentation-flotation earlier in time than according to McMaster counts, the day of the first positive sedimentation-flotation test was assumed to be first day of excretion).

[d] Sum of the *infection lag times* for all contact birds together.

Table 5
Start of oocyst excretion for inoculated and contact birds and oocyst output for different periods of experiments I-IV.

Exp	Inoculation dose I birds	Bird type	Start of excretion[e] Days pi (95% CI)	Oocyst output[f] Days 4-8 pi $Log_{10}AUC$ (95% CI)	Oocyst output[f] Days 9-13 pi $Log_{10}AUC$ (95% CI)	Oocyst output[f] Days 9-24 pi $Log_{10}AUC$ (95% CI)	Total oocyst output[f] Days 4-30 pi $Log_{10}AUC$ (95% CI)
I	5	I	5.00 (5.00-5.00)[a]	4.55 (4.31-4.80)[a]	5.19 (4.79-5.59)[a]	5.59 (5.27-5.91)[ab]	5.67 (5.41-5.93)[ab]
II	50	I	5.29 (4.86-5.73)[a]	4.06 (3.87-4.26)[b]	5.14 (4.78-5.49)[a]	5.31 (5.05-5.56)[a]	5.37 (5.14-5.60)[a]
III	500	I	4.60 (4.28-4.92)[ab]	5.44 (5.15-5.74)[c]	5.96 (5.65-6.26)[a]	5.98 (5.69-6.28)[b]	6.12 (5.86-6.39)[b]
IV	50,000	I	4.17 (3.84-4.49)[b]	6.18 (5.89-6.47)[d]	5.53 (5.31-5.75)[a]	5.53 (5.32-5.75)[ab]	6.29 (6.04-6.53)[b]
I	5	C	11.00 (10.27-11.73)[a]		4.72 (3.73-5.70)[a]	6.17 (5.98-6.37)[ab]	6.17 (5.98-6.37)[a]
II	50	C	10.56 (10.09-11.03)[ab]		5.31 (4.91-5.71)[a]	6.09 (5.88-6.31)[a]	6.09 (5.88-6.31)[a]
III	500	C	9.89 (9.67-10.11)[b]		6.67 (6.48-6.87)[b]	6.76 (6.58-6.94)[b]	6.76 (6.58-6.94)[b]
IV	50,000	C	9.67 (9.01-10.32)[b]		6.46 (5.77-7.15)[b]	6.83 (6.42-7.24)[b]	6.83 (6.42-7.24)[b]

Data represent means for I or C birds of each experiment and the associated 95% confidence intervals (CI), calculated based on the normal distribution.

[a-d] Values within the same column and concerning the same bird type (inoculated or contact birds) with different superscripts differ significantly ($P < 0.05$), based on linear regression analyses. The bonferroni corrected P values that were used were based on 16 comparisons, i.e. between I and I birds and C and C birds of the four different dose groups (six comparisons each) and between I and C birds within the same dose group (4 comparisons). Some comparisons could not be indicated in the table with superscripts, i.e. oocyst output during the second excretion peak (day 9-13 pi) was significantly higher for the 5, 50 and 500 groups than the first excretion peak (days 4-8 pi) according to a two sample paired t-test. Furthermore, linear regression analysis showed that oocyst output was significantly higher in contact birds compared to inoculated birds of the same dose group during days 9-24 pi.

[e] Mean of the start of oocyst excretion, based on the McMaster oocyst counting technique, expressed in days pi (where day pi = 0 is day of inoculation of I birds).

[f] Days 4-8 pi = first excretion peak I birds; Days 9-13 pi = second excretion peak I birds and first excretion peak C birds; Days 9-24 pi = period in which both I and C birds are comparable regarding exposure to infectious oocysts in the litter and are all actively shedding; Days 4-30 pi = total oocyst output for the entire duration of the experiment. $Log_{10}AUC$, calculated from daily OPG values per bird, was used as a measure for the oocyst output during the different periods.

Table 6
Results of the transmission rate calculations, including 95% confidence interval.

Group	Transmission rate[a]	95% Confidence Interval
5	0.58	0.29-1.03
50	0.67	0.40-1.03
500	0.81	0.40-1.42
50,000	0.94	0.60-1.77
Mean for all groups	0.70	

[a] Significant differences in transmission rates between dose groups were not found. Transmission rates calculated with lag times based on sedimentation-flotation results (*lag 2* in Tables 2 and 3) were 0.72 (95% CI 0.43-1.12) and 0.88 (95% CI 1.11-1.55) for the 50 and 500 oocyst group respectively and did not differ significantly from the McMaster count based calculations in this table.

These differences were significant for the 50,000 and 500 group compared to the 5 ($P = 0.04$ and $P < 0.032$ respectively) and 50 oocyst group (for both $P < 0.002$) in contact birds, and for the 50,000 and 500 group compared to the 50 oocyst group (for both $P < 0.002$) in inoculated birds (Table 5). Oocyst output was significantly higher in contact birds compared to inoculated birds of the same group for the period from days 9-24 pi ($P < 0.002$ for the 50, 500 and 50,000 oocyst group and $P = 0.018$ for the 5 oocyst group).

The transmission rate β varied between 0.58 and 0.94 per day for the different dose groups, though not significantly different between groups, and the overall estimate was 0.7 per day (Table 6). Transmission rates for the 50 and 500 oocyst group were slightly higher when sedimentation-flotation results were used to calculate *infection lag time* (*lag 2* in Tables 1-4), but these were not significantly different compared to the transmission rates based on McMaster counts (Table 6).

4. Discussion

The aim of this study was to quantify the effect of inoculation dose on oocyst excretion patterns and on subsequent infection dynamics of *E. acervulina* between broilers. A broiler infected with *E. acervulina* infected on average 0.7 susceptible contact chickens per day, which corresponds to an interval between exposure to an infected bird and contact-infection of approximately 34 hours. Birds inoculated with a high dose shed significantly more oocysts in the first days than those inoculated with lower doses, which was consistent with previous studies (Williams, 2001). Despite these significant differences, only small, non-significant differences in transmission rates were found between dose groups. This finding implies that a low dose is sufficient to establish an infection, and that the number of oocysts in faecal samples not unequivocally provides a suitable variable to determine differences in infectiousness and subsequent transmission. Possibly, the amount of infectious faecal

droppings in the environment, rather than the number of oocysts in these droppings, determined the transmission rate in these experiments.

In our experiments a dose of only 5 oocysts was sufficient to establish infection in inoculated birds, which was consistent with a previous study (Norton and Joyner, 1986), but whether contact-infection can be established upon exposure to birds that had a low grade infection, was not studied to this date. It had been shown that a 10-fold increase of inoculation dose roughly results in a 10-fold increase in oocyst output until the crowding threshold is reached (Williams, 2001). Based on the large differences in oocyst output, the inoculation dose was expected to significantly affect the transmission rate, although not known to which level. Our experiments have shown, however, that transmission rates were not significantly influenced by the amount of oocysts shed in the environment despite the observed differences in oocyst levels. This is counterintuitive and suggests that drawing conclusions about transmission from shedding patterns is not straightforward.

Oocyst output was significantly higher in contact birds compared to inoculated birds of the same group for the period from days 9-24 pi, in which inoculated and contact birds were exposed to similar numbers of oocysts from the environment. This finding suggests that inoculation may have induced an immune response that reduced oocyst output upon re-infection as previously reported (e.g. Lillehoj, 1988, Williams, 1995b, Blake et al., 2005).

Inoculated birds of the 5, 50 and 500 oocyst group had a high second peak in shedding around days 11-13 pi, which was absent in the 50,000 oocyst group. The higher inoculation dose possibly induced a more effective immune response, as proposed by Brackett and Bliznick (1952). Another explanation is a lack of intestinal epithelial cells available for parasitisation, i.e. "the crowding effect" (Williams, 2001) and even the higher age of the birds at inoculation in experiment IV might also have played a role. Further research is needed to confirm and quantify the influence of the immune response on oocyst output and transmission of the parasite.

Our findings suggests that in a large flock a very low initial dose of oocysts can establish a low grade infection in naïve birds, which can quickly be transmitted to other birds in the flock resulting in higher oocyst outputs in contact-exposed chickens. Contact birds, that have escaped infection in the beginning of the infection cycle in the flock, might therefore be exposed to high infectious doses, which may lead to severe symptoms depending on host and environmental factors and the time of infection during the production cycle (Henken et al., 1994, Graat et al., 1996, Klinkenberg and Heesterbeek, 2007). This illustrates that challenge experiments alone might not provide sufficient insight in the course of infection within a flock and that transmission studies in pairs and groups should contribute to knowledge about this disease. It should be mentioned, however, that it is not yet clear whether infection dynamics in a commercial poultry house is comparable to the outcome in our experiments. Experiments with groups of birds are necessary to study transmission of *Eimeria* species in flocks. Another difference is that we used of SPF birds to reduce the influence of factors other than inoculation dose on transmission. Although SPF birds differ in many aspects from

conventional birds, e.g. in the presence of maternal antibodies, possible effects on susceptibility to infection are most likely negligible, as birds were already a few weeks old and protective immunity against infection is mostly caused by T-cell mediated immunity (Lillehoj and Lillehoj, 2000).

Despite differences between commercial flocks and pair-wise experiments, we consider our experiments as a first step towards unravelling part of the complex transmission dynamics between birds. This can be helpful for designing experiments to study infection dynamics in populations. The influence of previous exposure to oocysts or other factors, that might influence infection dynamics and the course of an *Eimeria* species infection in a flock, remains to be determined.

Acknowledgements

Appendix A.

A.1. Estimation of probability of infection and transmission rate
A precise method to estimate the probability of infection, p, and subsequently the transmission rate, including confidence intervals, is based on the probability distribution of p, which can be calculated from the experimental data as explained below.

The data in pairs in which the contact bird became infected follow a negative binomial distribution, with the observed number of "failures", $\underline{x}$, i.e. the *infection lag time* in days before successful transmission has taken place as variable, and with the probability p of success for each observed day as parameter to be estimated.

The parameters and variables that are used for these calculations are:

- n_k = number of pairs (i.e. each consisting of one inoculated and one contact bird);
- n_x = number of observed days: from day 8 pi onwards (first day at which contact birds are assumed to be at risk of becoming infectious, i.e. at risk for starting to excrete oocysts, as explained below) until the end of the experiment (for experiments I - III until day 29 pi: n_x = 22 days, for experiment IV until day 25 pi: n_x = 18 days);

And the following stochastic variables are observed during the experiment:

- k =   *total number of infected contact birds* given in Tables 1-4 (i.e. successful transmissions from inoculated to contact bird);
- x =   *infection lag time*, which is defined as the number of days between the first day when it is considered possible for a contact bird to start its infectious period and the day when the contact bird actually started to excrete oocysts and had become an infected case (Tables 1-4). When contact birds first showed positive McMaster oocyst counts, a successful transmission event had taken place and birds were considered to have become infectious. The first day at which contact birds could become infectious was assumed to be day 8 pi (prepatent period of inoculated birds + prepatent period of contact birds), since the prepatent period for both inoculated and contact birds was assumed to be four days (Edgar, 1955, Joyner and Long, 1974, McDougald, 2003). Sporulation time of oocysts was assumed to be negligible since the first sporulation can occur within hours of excretion (Graat et al., 1994). The *infection lag time* was based on the biological information of the parasite as explained above, and is therefore independent of limitations of diagnostic tests and small variations between birds, which allows for a straightforward comparison of transmission rates between inoculation dose groups. The assumption that day 8 pi was the first possible day of excretion for contact birds was confirmed by the data in Tables 1-4, as one of the contact bird started output at day 8 pi;
- y =   number of days that had passed ("failures") before reaching k successful transmissions, i.e. the x added over all k pairs ($\sum x_i$, i.e. the *total infection lag time* Tables 1-4).

As the estimator for the probability of infection, $\widehat{p}$, will be a function of two stochastic variables, i.e. k and y, we first derived the joint probability distribution of these two variables. The probability that transmission occurs in k out of n_k pairs, where the experiment is ended after n_x days is:

$$prob[\underline{k} = k] = \binom{n_k}{k} \left((1-p)^{n_x}\right)^{n_k - k} \left(1 - (1-p)^{n_x}\right)^k \qquad \text{(A.1)}$$

Given that transmission occurs in k pairs, the variable y is the *infection lag time* for all k pairs cumulated and was calculated from the data in Tables 1-4. As these y failures can be divided over the total number of pairs where successful transmission has taken place, k, the negative binomial distribution describes the distribution of y failures before k successes:

$$prob[\underline{y} = y] = \binom{y+k-1}{k-1} \left(p^k (1-p)^y\right) \qquad \text{(A.2)}$$

This formula is an approximation, as in fact the number of failures before a success can never be larger than n_x.

In a paper by Gan and Bain (1998) the general derivation for censored sampling from the geometric distribution is given. In this paper it is shown that indeed $\underline{k}$ (in their notation W) and $\underline{y}$ (in their notation S_W) are sufficient statistics for p . They give the probability generating function (*pgf*) for the joint distribution for these two stochastic variables in Eq. (3.2) in their paper. To derive from that *pgf* to a probability mass function (*pmf*), approximations are given. Here we use another approximation by observing that if $n_x \rightarrow \infty$ (in their notation $n_x = x_0+1$), the *pgf* becomes (in our notation):

$$G_{k,y}(t_1, t_2) = \left(\frac{p}{1 - qt_2} \right)^{n_k} t_1^{n_k} \tag{A.3}$$

Hence, the joint *pmf* is the product of a negative binomial distribution, with number of successes equal to n_k, as in all pairs contact-infection will have occurred, and the degenerated delta function. Therefore, if for the approximation the n_x is sufficiently high, so that we almost always observe contact-infections in each pair, the joint distribution of $\underline{k}$ and $\underline{y}$ is given by:

$$prob[\underline{y} = y, \underline{k} = k \mid p, n_x, n_k] = prob[\underline{k} = k \mid p, n_x, n_k] * prob[\underline{y} = y \mid p, k] \tag{A.4}$$

where the probability of infection, p , is the parameter that has to be estimated.

$$\frac{\partial prob[\underline{y} = y, \underline{k} = k \mid p, n_x, n_k]}{\partial p} = 0 \tag{A.5}$$

The maximum likelihood estimator is given by maximizing Eq. (A.4) by setting the derivative of the distribution in Eq. (A.5) with respect to p to zero and then solving Eq. (A.5) for p . Thus we arrive at the following maximum likelihood estimator for the probability of infection:

$$\underline{\hat{p}} = \frac{\underline{k}}{\underline{y} + \underline{k} + (n_k - \underline{k})n_x} \tag{A.6}$$

which is the same expression, apart from notational differences, as Eq. (2.2) in the paper by Gan and Bain (1998). Note that the estimator $\widehat{p}$ only depends on the total number of "failures" ($\underline{y}$ and not on the separate x_i values). With the maximum likelihood estimation for $\widehat{p}$ the transmission rate can be estimated with:

$$\widehat{\beta} = -2\ln(1 - \widehat{p}) \tag{A.7}$$

A.2. Calculation of the confidence interval for the transmission rate

To derive the 95% confidence interval (95% CI) for the estimation of the probability of infection, $\hat{p}$, the cumulative distribution function of this estimator (based on Eq. (A.4)) was used. The lower limit of the 95% CI for $\underline{\hat{p}}$ was found by solving p from the equation:

$$prob\left(\forall_{\underline{k},y}\,\hat{\underline{p}} \le p\right) = 0.975 \qquad (A.8)$$

In words, we searched for the value of p for which the probability that the (stochastic) estimator is smaller than the actual estimate, is 97.5%. Thus, we defined the critical region to be all ($\underline{k}$, $\underline{y}$) values for which the estimator is smaller than the actual estimate from our own data. Similarly, the upper limit of the 95% CI for $\underline{\hat{p}}$ was found by solving p from the equation:

$$prob\left(\forall_{\underline{k},y}\,\hat{\underline{p}} \ge p\right) = 0.975 \qquad (A.9)$$

With these values for $\hat{p}$ the lower and upper 95% CI limit for the transmission rate β could be calculated with Eq. (A.7). All calculations were carried out using Wolfram Mathematica 6.0.0 (Wolfram, 2007).

A.3. Differences between transmission rates

To test if significant differences in transmission rates between the four dose groups were present, six pair-wise comparisons were carried out, using the null-hypotheses that the probability of infection was equal between groups:

$$H_0 : p_1 - p_2 = 0$$

$$H_1 : p_1 - p_2 \ne 0$$

As a test statistic we used the difference between the estimated p 's:

$$\delta = \left|\hat{p}_1 - \hat{p}_2\right|$$

The *P* value is the probability

$$prob\left[\underline{\delta} \ge \left|\hat{p}_1 - \hat{p}_2\right|\right]$$

under the null-hypothesis.

For each comparison we calculated the distribution of δ under the null-hypothesis by assuming that $p = \left(\hat{p}_1 + \hat{p}_2 \right) / 2$. As computational difficulties occur when these calculations are carried out for all possible combinations of $\underline{k}$ and $\underline{y}$, only part of the critical region was used. Therefore, the *P* value for the difference between groups under the null-hypothesis is underestimated in our calculations. As the null-hypothesis was accepted in all cases and the overall *P* value was more than 0.20, this underestimation is not important, as these *P* values can be considered as a lower bound to the real significance level. Therefore it is concluded that transmission rates between groups did not differ significantly.

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Chapter 4

Effect of *Eimeria acervulina* infection history on the immune response and transmission in broilers

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Abstract - Heterogeneity in exposure to *Eimeria* species of chickens in a flock will result in differences between individual birds in oocyst output and acquired immunity, which subsequently affects transmission of the parasite in the population. The aim of this study was to quantify effects of previous infection of broilers with *Eimeria acervulina* on immune responses, oocyst output and transmission. A transmission experiment was carried out with pair-wise housed broilers, that differed in infection history. This "infection history" was achieved by establishment of a primary infection by inoculation of birds with 50,000 sporulated *E. acervulina* oocysts at day 6 of age ("primed"); the other birds did not receive a primary infection ("naïve"). The actual transmission experiment started at day 24 of age: one bird (*I*) was inoculated with 50,000 sporulated oocysts and was housed together with a non-inoculated contact bird (*C*). Oocyst excretion and parameters describing transmission, i.e. the number of infected *C* birds and time passed before start of excretion of *C* birds, were determined from day 28 to day 50 for six pairs of four different combinations of *I* and *C* birds (*I-C*): naïve-naïve, naïve-primed, primed-naïve and primed-primed. Immune parameters, CD4$^+$, CD8$^+$, αβTCR$^+$ and γδTCR$^+$ T cells and macrophages in duodenum, were determined in an additional 25 non-primed, non-inoculated control birds, and in the naïve-naïve and naïve-primed groups, each group consisting of 25 pairs. Although the numbers of CD4$^+$ T cells and γδTCR$^+$ T cells increased after primary infection, none of the immunological cell types provided an indication of differences in infectivity, susceptibility or transmission between birds. Oocyst output was significantly reduced in primed *I* and *C* birds. Transmission was reduced most in the primed-primed group, but nonetheless transmission occurred in all groups. This study also showed that acquired immunity significantly reduced oocyst output after inoculation and contact-infection, but not sufficiently to prevent transmission to contact-exposed birds.

Keywords - *Eimeria acervulina;* broilers; transmission; immunity; infection dynamics; immuno-epidemiology

1. Introduction

One of the most prevalent infections in commercial poultry is coccidiosis, caused by various species of the genus *Eimeria*. Infection with *Eimeria* species may cause enteritis, mortality, welfare problems and economic losses due to production losses and costs for treatment or prevention (Shirley et al., 2005). The majority of the current control strategies rely on the use of anticoccidials, supplemented with live attenuated vaccines. Increasing resistance among *Eimeria* species (Chapman, 1997; Peek and Landman, 2003), legislative restrictions against in-feed medication, and the lack of success in producing cost-effective vaccines, stimulate the demand for alternative approaches of the problem. More research, aimed at increasing insight in processes that result in a protective immune response and towards providing adequate indicators of protection against infection, might contribute to the development of alternative intervention strategies.

Immune responses, developed after (repeated) infection with parasites such as *Eimeria* species affect the susceptibility and infectivity of individual birds (Rose, 1987; Lillehoj, 1988; Stiff and Bafundo, 1993; Williams, 1995; Claerebout and Vercruysse, 2000). As a result of differences in the exposure of birds to infectious oocysts from the environment, birds in a group are likely to differ in their infection history, which will result in a heterogeneous population with respect to infectivity and immunity. This heterogeneity will cause variability between birds with respect to oocyst excretion and their response after subsequent ingestion of oocysts in the population, which implies that host-pathogen interactions in individuals will affect the transmission of the parasite between individuals in a population and vice versa (Severins et al., 2007).

Most studies carried out in the last decades focused either on immunity (Lillehoj, 1988; Stiff and Bafundo, 1993; Williams, 1995) or epidemiology (Graat et al., 1998; Williams, 1998), but it is the mutual interaction between host and pathogen ("within-host dynamics"), and between infectious and susceptible hosts in a flock and the environment ("between-host dynamics") that determines the dynamics of the infection with the pathogen in a flock. Although this seems straightforward, the outcome of these interactions may give rise to non-linear effects, which may result in a counterintuitive and an unpredictable course of the infection (Roberts and Heesterbeek, 1995, 1998; Graat et al., 1996; Klinkenberg and Heesterbeek, 2007). Therefore, to provide more insight in the course of the disease coccidiosis in a flock, the dynamics of the immune reaction in individual hosts and transmission of the infection between hosts should be studied together.

The aim of the current study was to examine the relation between immune responses and transmission between birds. A transmission experiment was carried out with broilers with different (artificially induced) infection histories to *Eimeria acervulina*, presumed to result in different levels of acquired immunity. Oocyst output kinetics, intestinal immune responses in individual birds and transmission characteristics were quantified to determine whether immune parameters correlated with infectivity or susceptibility of birds, which would be

useful to characterise differences between individual hosts, and to monitor and predict transmission of infection between birds. The approach described in this paper might also be applied to other (parasitic or protozoan) diseases.

2. Materials and methods

2.1. Chickens and management
At day 0 of age (day 0 of the experiment), 173 male SPF broiler chickens were obtained from GD-Animal Health Service (AHS, Deventer, the Netherlands). The chicks originated from a specified pathogen free (SPF) parent flock, a crossbred of Cobb, Hybro and Ross, kept in an *Eimeria* species free environment. Until day 25 of age birds were housed in groups in battery cages (Tecniplast®, Tecnilab-BMI, the Netherlands). From day 25 onwards, chicks were housed in pairs on wood shavings in randomly allocated floor pens (height x width x depth = 80 x 60 x 50 cm). Control birds were housed in groups of five birds in battery cages on wood shavings.

Room temperature was 32 °C at day 0 and was gradually decreased to 18 °C at day 50. The chicks were subjected to a lighting scheme of 23 hours of light per day. A broiler ration (2900 kcal/kg of metabolisable energy) without anticoccidial drugs, and drinking water were available *ad libitum*.

The birds were observed twice daily for signs of illness or welfare impairment. Birds were housed, handled and treated following approval by the Animal Experiments Committee of Utrecht University (Utrecht, the Netherlands), in accordance with the Dutch Experiments on Animals Act.

2.2. Experimental design
An experiment was carried out with pairs of birds consisting of one inoculated (*I*) and one non-inoculated contact (*C*) bird, referred to as a pair-wise transmission experiment (Velthuis et al., 2007). The transmission experiment started when the birds were 24 days old with inoculation of one bird per pair (*I*). Seventy-four *I* birds were orally inoculated at day 24 with 50,000 sporulated *E. acervulina* oocysts (Weybridge W119, supplied by AHS; originally provided by the Central Veterinary Laboratory in Weybridge, United Kingdom), suspended in 1 ml of tap water. The 74 contact birds (*C*) were not inoculated. *I* and *C* birds were housed pair-wise in a floor pen from day 25 onwards until the end of the experiment. Transmission of the infection was subsequently measured by determining for each pair if and when the contact bird became infected, based on oocyst excretion.

At the start of the transmission trial, birds differed in infection history, resulting in different immune status. The infection history was induced by artificial inoculation of 49 birds at day 6, with a single oral dose of 50,000 sporulated *E. acervulina* oocysts. At that age, these birds received a primary infection, denoted by "*P*" and referred to as "primed".

The other birds did not receive such a primary infection (denoted by "*N*" and referred to as "non-primed" or "naïve"). From day 6 to day 24 of age, birds treated equally were housed together in battery cages.

The transmission experiment was carried out with pairs of birds in four different combinations:

- Group 1, "*NN*", Naïve inoculated bird and naïve contact bird (I_{NN} and C_{NN} bird);
- Group 2, "*NP*", Naïve inoculated bird and primed contact bird (I_{NP} and C_{NP} bird);
- Group 3, "*PN*", Primed inoculated bird and naïve contact bird (I_{PN} and C_{PN} bird);
- Group 4, "*PP*", Primed inoculated and primed contact bird (I_{PP} and C_{PP} bird).

Note that for the group notation the abbreviation for the infection status of the inoculated bird, "*N*" or "*P*", precedes that of the contact bird. For individual birds, the notation starts with the status in the transmission experiment, i.e. either inoculated, "*I*" bird or non-inoculated contact, "*C*" bird, followed by a subscript to denote to which group they belong, i.e. *NN*, *NP*, *PN* or *PP*.

Transmission and oocyst excretion were quantified for all four groups, consisting of six pairs each. Immune parameters were quantified, based on a study by Cornelissen et al. (2009), for Groups 1 and 2 (*NN* and *NP* respectively), using 25 additional pairs each; five birds were sacrificed for quantification of immune parameters at each of the days 28, 31, 33, 36 and 39. For this immunological study we also included another five groups of five non-inoculated, non-primed chickens that served as "negative controls". Group composition and treatments are explained in Table 1 and illustrated with a flow chart in Fig. 1.

2.3. Immune parameters

Five pairs of the *NN* and *NP* group and five birds of the control group were killed by cervical dislocation at days 28, 31, 33, 36 and 39 (Table 1). One cm of the duodenal loop was snap-frozen in liquid nitrogen and stored at -70 °C until further examination by immunohistochemistry. Immunohistological staining by an indirect immunoperoxidase method was performed on frozen duodenum sections (8 µm thick), collected from chickens of 28, 31, 33, 36 and 39 days of age as described by Cornelissen et al. (2009). In short, endogenous peroxidase was inhibited and were subsequently incubated for one hour with monoclonal antibodies against $CD4^+$ T cells (1:200; CT-4, Southern Biotech), $CD8^+$ T cells (1:200; CT-8, Southern Biotech), $\alpha\beta TCR^+$ T cells (1:50; TCR2, Southern Biotech), $\gamma\delta TCR^+$ T cells (1:400; TCR1, Southern Biotech) or monocytes/macrophages (1:50; KUL-01, Southern Biotech) followed by peroxidase-conjugated rabbit anti-mouse *Ig* (1:80; P0161, Dakopatts, Denmark). Peroxidase activity was detected by 0.05% 3,3-diaminobenzidine (DAB) in 0.1 M Tris-HCl solution (pH 7.5) containing 0.03% H_2O_2. The images were analysed with Image-Pro Plus (version 5.1, media cybernetics) to quantify the number of stained T cells per 0.5 mm^2.
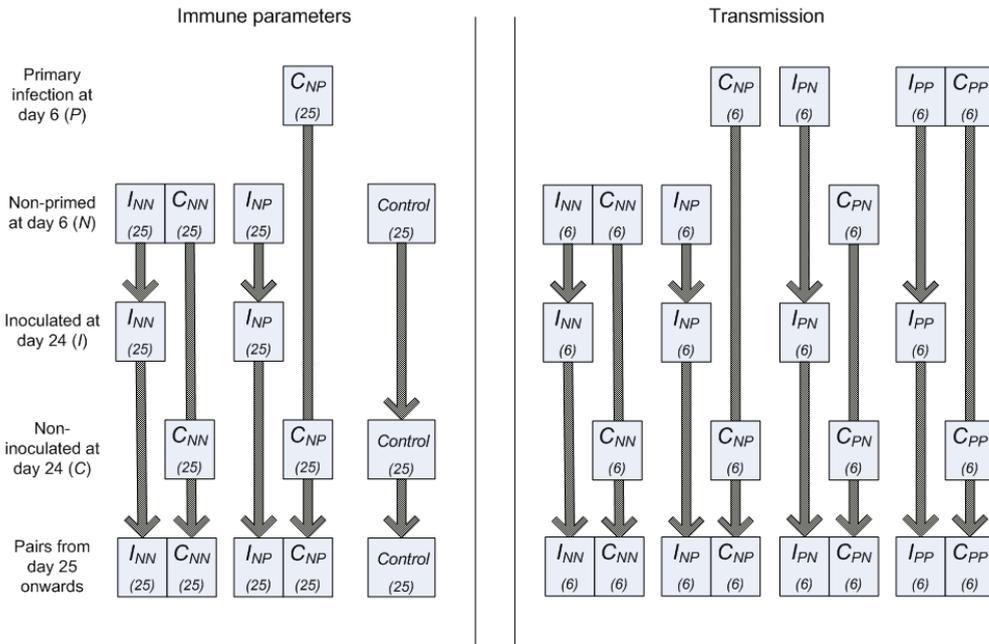
Fig. 1. Flow chart of the study design, illustrating the treatments at different times during the experiment. At day 6, birds either received a primary infection (*P*) or not (*N*) to induce different infection histories and were inoculated (*I*) or not (*C*) at day 24. From day 25 onwards, four different combinations of *I* and *C* birds were housed pair-wise in floor pens. Immune parameters (left side of the flow chart) were determined for two groups and transmission parameters (right) for four different groups. The notations for the different bird types in the experiment (I_{NN}, C_{NN}, I_{NP}, C_{NP}, I_{PN}, C_{PN}, I_{PP}, C_{PP} and control birds) are given with the number of birds between brackets.

Per bird five images were acquired and analysed and the mean of the five $\log_{10}$-transformed T cell counts per bird was used for the statistical analysis.

Immunological data was analysed per group to determine whether elevated or decreased levels of the different cell types could be identified as indicators of infection or acquired/protective immunity. Therefore, a linear mixed model was applied using SAS for Windows 9.1 (SAS Institute Inc., Cary, NC, USA) with $\log_{10}$-transformed mean cell counts as dependent variable and litter cage as random factor. Bird type (five different bird types, i.e. I_{NN}, C_{NN}, I_{NP}, C_{NP} and control birds), day (days 28, 31, 33, 36 and 39) and the interaction group*day were entered into the model as explanatory variables. The two-tailed partial F-test (type III) was used as the elimination criterion for the model building and the fit of the model was assessed by the Akaike's Information Criterion. The best model for $CD4^+$ and $CD8^+$ T cells consisted of day and the interaction between group*day. The final model for $\alpha\beta TCR^+$ and $\gamma\delta TCR^+$ cells contained day, group and group*day as explanatory variables. Differences in T cell counts were assessed per day between *I* birds of both groups and control birds, between *C* birds of both groups and control birds, between I_{NN} and I_{NP} birds and between C_{NN} and C_{NP} birds.

Table 1
Experimental design: composition and notation of groups, inoculation and measurements.

Group	Bird	Inoculation day 6 (primary infection)[a]	Status at day 24[a]	Inoculation day 24[b]	# of chicks[c]	Parameters tested[c]
Control	Control	None	Naïve	Non-inoculated	25	Immune parameters
1: *NN*	I_{NN}	None	Naïve	Inoculated	6	Transmission/oocyst output
					25	Immune parameters
1: *NN*	C_{NN}	None	Naïve	Non-inoculated	6	Transmission/oocyst output
					25	Immune parameters
2: *NP*	I_{NP}	None	Naïve	Inoculated	6	Transmission/oocyst output
					25	Immune parameters
2: *NP*	C_{NP}	Inoculated	Primed	Non-inoculated	6	Transmission/oocyst output
					25	Immune parameters
3: *PN*	I_{PN}	Inoculated	Primed	Inoculated	6	Transmission/oocyst output
3: *PN*	C_{PN}	None	Naïve	Non-inoculated	6	Transmission/oocyst output
4: *PP*	I_{PP}	Inoculated	Primed	Inoculated	6	Transmission/oocyst output
4: *PP*	C_{PP}	Inoculated	Primed	Non-inoculated	6	Transmission/oocyst output

[a] Before start of the transmission experiment at day 24, the birds were either primed/had received a primary infection (i.e. were inoculated at day 6 with 50,000 sporulated *E. acervulina* oocysts) to artificially induce infection history, or were naïve/non-primed (i.e. were not inoculated at day 6).
[b] At day 24 birds were either inoculated (*I* birds) or not (*C* birds and negative control birds) to start the infectious process in the pair-wise transmission experiment.
[c] Oocyst output and transmission were determined for *I* and *C* birds for six pairs of Groups 1-4 from days 28-50. Immune parameters were determined in 25 control birds and an additional 25 pairs of *I* en *C* birds from Groups 1 (*NN*) and 2 (*NP*) at days 28, 31, 33, 36 and 39. Five birds of each group were used per day of testing.

Bonferroni corrected pair-wise multiple *t*-tests were carried out to test the difference between each above mentioned pair of means. Model assumptions were evaluated by examining normality and equality of variances of the residuals. The level of statistical significance was set at $P < 0.05$.

2.4. Droppings collection, processing and oocyst counts

The number of oocysts per gram of faeces (OPG) was quantified using data from six pairs of birds from Groups 1-4. Single individual droppings were collected daily from days 10 to 23 from primed birds and daily from days 28 to 50 from all *I* and *C* birds, or until no oocysts were detected for three consecutive days. The single droppings were collected and analysed according to a procedure described by Velkers et al. (2010). Briefly, each chick was placed in a cardboard box with a clean paper sheet for one to two hours. The single dropping was weighed and OPG was determined, according to a modification of a McMaster oocyst counting chamber technique described by Long and Rowell (1958).

The detection limit of the McMaster technique was 83 oocysts per g of faeces for a faecal sample of 4 g. If no oocysts were found, a modification of the sedimentation-flotation (SF)

technique was applied (Long et al., 1976), which is a more sensitive technique, though only qualitative (Mes, 2003).

For the oocyst excretion patterns, the average oocyst production was determined by calculating the mean of the $\log_{10}$-transformed OPG per day for I and C birds per group. As a measure for the total number of excreted oocysts, the area under the curve (AUC) of oocyst output was calculated for each bird from the daily non-transformed OPG results. AUC was $\log_{10}$-transformed, to obtain normally distributed data, and the mean $\log_{10}$-transformed AUC ($\log_{10}$ AUC) was calculated for I and C birds per group. Also the period in which OPG was intermittently above zero (last day of positive OPG minus first day of positive OPG results = PERIOD) and the highest measured $\log_{10}$ OPG peak output (PEAK) were determined.

These data were analysed per group to determine how infection history influences oocyst excretion characteristics. Therefore, a linear mixed model was applied with $\log_{10}$ AUC, √PERIOD or PEAK as dependent variable, bird type (eight different bird types, i.e. I and C birds of each of the four different groups) as fixed factor and litter cage as random factor using SAS for Windows 9.1 (SAS Institute Inc., Cary, NC, USA). The two-tailed partial F-test (type III) was used as the elimination criterion for the model building and the fit of the model was assessed by the Akaike's Information Criterion. Bonferroni corrected pair-wise multiple t-tests were carried out to test the difference between each pair of means. Model assumptions were evaluated by examining normality and equality of variances of the residuals. The level of statistical significance was set at $P < 0.05$.

2.5. Transmission between birds

Transmission of the parasite was quantified for Groups 1-4 using the following measures:

- total number of infected contact birds per group;
- infection delay time per pair of birds and the total infection delay time per group.

The infection delay time, calculated per pair of birds, is defined as the number of days between the first day at which a contact bird could have started excreting oocysts and the actual day oocysts were detected for the first time. The first day at which contact birds could have started shedding oocysts was two prepatent periods after inoculation (day 32), assuming sporulation time of oocysts to be negligible (Graat et al., 1994). The prepatent period for *E. acervulina* is approximately four days (Edgar, 1955; Joyner and Long, 1974; McDougald, 2003).

The day the contact bird actually started to excrete oocysts was based either solely on the McMaster oocyst counts (McM), or on combined McMaster and sedimentation-flotation test results (McM/SF). The sedimentation-flotation technique was more sensitive but did not allow quantification of output. When output started earlier according to the sedimentation-flotation test, this day was considered first day of excretion. Infection delay times were added for all pairs per group to obtain the total infection delay time.

3. Results

3.1. Immunohistochemistry

Numbers of T cells in birds at days 28, 31, 33, 36 or 39 are shown in Fig. 2. T cell counts did not differ significantly between non-primed C_{NN} and primed C_{NP} birds. C_{NN} birds had significantly higher $CD4^+$ T cell counts compared to the control birds on day 33. Non-significant increases of $CD4^+$ T cells were observed in I birds on days 31, 33, and 39 (I_{NN}) and day 33 (I_{NP}), and in C_{NN} birds on day 39. C_{NP} birds showed no marked increase in their intestinal $CD4^+$ T cell amount compared to control birds. The number of $\gamma\delta TCR^+$ T cells in I_{NN} and I_{NP} birds showed a steep increase at day 31, and remained high until day 39 (I_{NN}) and 36 (I_{NP}). The number of $\gamma\delta TCR^+$ T cells of C_{NN} birds increased at day 36, whereas the primed C_{NP} birds did not show a marked increase. Significant differences in $CD8^+$ T cells and macrophages were not observed between inoculated and contact birds of the NN and NP groups when compared to control birds. The number of $\alpha\beta TCR^+$ T cells in control birds showed high fluctuations for all observed days (data not shown).

3.2. Oocyst excretion patterns and transmission of infection

After priming, the mean total oocyst excretion was $\log_{10} AUC = 6.44$ (S.D. 0.26). The excretion data after inoculation or contact-infection are summarised in Table 2. Supplementary Tables 4-7 show oocyst counts and the associated infection delay times; in Table 3 the total infection delay time and the total number of infected contact birds are given per group.

All non-primed I and C birds (I_{NN}, I_{NP}, C_{NN}, C_{PN}) excreted detectable numbers of oocysts (Tables 2 and Supplementary Tables 4-6). Oocyst output in the primed I_{PN} birds was detected in only four birds, after day 42 of the experiment (Supplementary Table 6), whereas all non-primed C_{PN} birds excreted oocysts. Six primed C_{NP} birds showed oocyst output (five birds positive according to McMaster counts and six according to sedimentation-flotation results) (Supplementary Table 5). Five primed I_{PP} birds were observed to excrete oocysts (one according to McMaster counts and five birds according to sedimentation-flotation results), whereas the bird that did not show oocyst excretion according to both tests was the only one with its pen mate positive (Supplementary Table 7).

Non-primed I and C birds shed oocysts significantly longer, and had a higher $\log_{10} AUC$ and peak output compared to primed I and C birds (Table 2). The oocyst excretion parameters were significantly higher for C_{PN} birds compared to C_{NP} birds and were lowest for C_{PP} birds. I_{PN} birds had a significantly longer OPG positive period and higher $\log_{10} AUC$ than I_{PP} birds (Table 2).

Oocyst output characteristics, e.g. period of excretion, total and peak output, were comparable between non-primed I and C birds, irrespective of group (Table 2). Primed I_{PN} and C_{NP} birds showed comparable oocysts output characteristics and also primed I_{PP} and C_{PP} birds were not significantly different from each other regarding output data (Table 2).
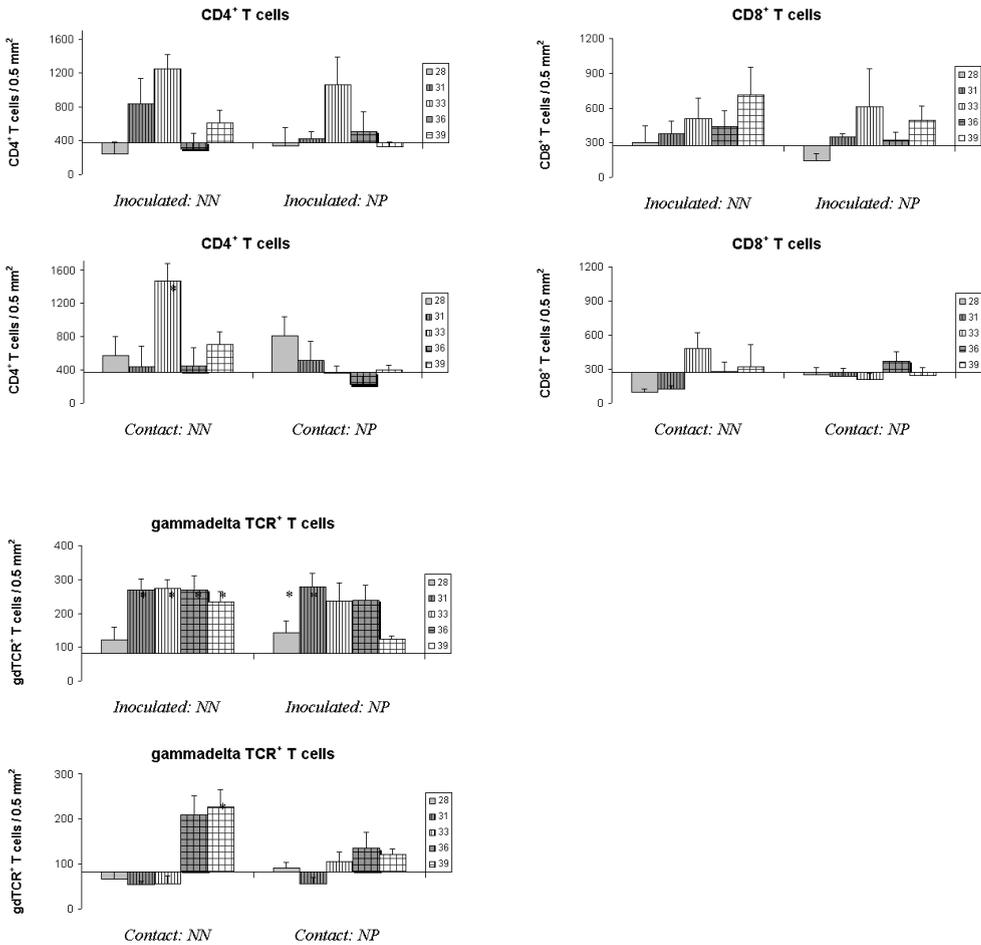
Fig. 2. The number of CD4^{+}, CD8^{+} and γδTCR^{+} cells in duodenum sections from inoculated and contact chickens of the *NN* group and the *NP* group compared to a baseline formed by chickens of the control group at 28, 31, 33, 36 and 39 days of age. CD4^{+}, CD8^{+} and γδTCR^{+} T cells were stained and the amount of positive cells in an intestinal segment was counted. Mean of five chickens is shown. Baseline of the graphs is not set at zero, but at the mean amount of the control values. * Significant differences in the numbers of the different T cell types between birds of the experimental group and control birds ($P < 0.05$). Note the difference in the scales. Plain columns represent measurements before transmission of the parasite to the contact bird could have taken place, striped columns represent data when transmission between inoculated and contact birds could have taken place and checked columns represent data when both birds are able to infect each other.

Table 2
Oocyst output characteristics for *I* and *C* birds for all groups.

Group	Bird	# of excreting birds (McM)[1]	# of excreting birds (McM/SF)[1]	OPG positive period Mean # of days (S.D.)[2]	Total oocyst output Mean log$_{10}$ AUC (S.D.)[3]	Peak oocyst output Mean log$_{10}$ OPG (S.D.)[4]
1: *NN*	I_{NN}	6	6	14.67 (3.93)[a]	6.29 (0.30)[a]	5.82 (0.26)[a]
1: *NN*	C_{NN}	6	6	13.83 (0.98)[a]	6.83 (0.51)[a]	6.57 (0.53)[a]
2: *NP*	I_{NP}	6	6	13.00 (3.10)[a]	6.36 (0.39)[a]	6.03 (0.51)[a]
2: *NP*	C_{NP}	5	6	6.00 (5.76)[b]	2.46 (1.42)[b]	2.18 (1.21)[b]
3: *PN*	I_{PN}	4	4	2.17 (3.43)[b]	1.59 (1.40)[b]	1.63 (1.44)[bc]
3: *PN*	C_{PN}	6	6	14.50 (2.17)[a]	5.52 (0.47)[a]	5.23 (0.44)[a]
4: *PP*	I_{PP}	1	5	1.00[b,5]	0.49 (1.20)[c]	0.48 (1.18)[c]
4: *PP*	C_{PP}	1	1	1.00[b,5]	0.24 (0.58)[c]	0.23 (0.57)[c]

The mean of the OPG positive period, total oocyst output and peak oocyst output, followed by the standard deviation (S.D.) between brackets, is given per bird type for each group.
[1] Number of birds excreting oocysts based solely on McMaster oocyst counts (McM) or on combined McMaster and sedimentation-flotation test results (McM/SF).
[2] Mean of the OPG positive period (in number of days) of (intermittent) oocyst excretion, which is the last day with OPG larger than zero minus the first day with OPG larger than zero.
[3] Mean of the log$_{10}$-transformed AUC of the OPG, which represents total oocyst output during the entire oocyst excretion period.
[4] Mean of the highest log$_{10}$-transformed OPG, which represents the peak of oocyst output.
[5] Only one I_{PP} and one C_{PP} bird were positive, therefore the standard deviation could not be calculated.
[a-c] Values within the same column and with different superscript letters differ significantly ($P < 0.05$).

In Fig. 3 mean oocyst excretion patterns, based on McMaster oocyst counts, are visualised for the four groups. Start of oocyst output in C_{NP} birds was not delayed (day 33, Fig. 3B) compared to the C_{NN} birds (Fig. 3A) but remained low for the duration of the experiment. On days 33-36 the C_{NP} birds excreted a lower number of oocysts than the I_{NP} birds, whereas these birds have experienced the same environment and infection history in numbers of oocysts (50,000 in a single dose at inoculation on day 24 for I_{NP} birds and the same dose at priming on day 6 for C_{NP} birds). Oocysts were detected in faeces of C_{NN} and C_{PN} birds from day 33 onwards (Supplementary Tables 4 and 6). The peak of the latter was approximately six days later. In the *PP* group oocyst output was hardly detectable.

The infection delay times in the *NP* group were highly variable between pairs and were reduced when based on sedimentation-flotation results compared to McMaster counts (Supplementary Table 5). The lowest total infection delay time was found in the non-primed *NN* group (10 days) and the highest total infection delay time and lowest number of infected contact birds (one infected contact bird) was found in the primed *PP* group (Table 3).
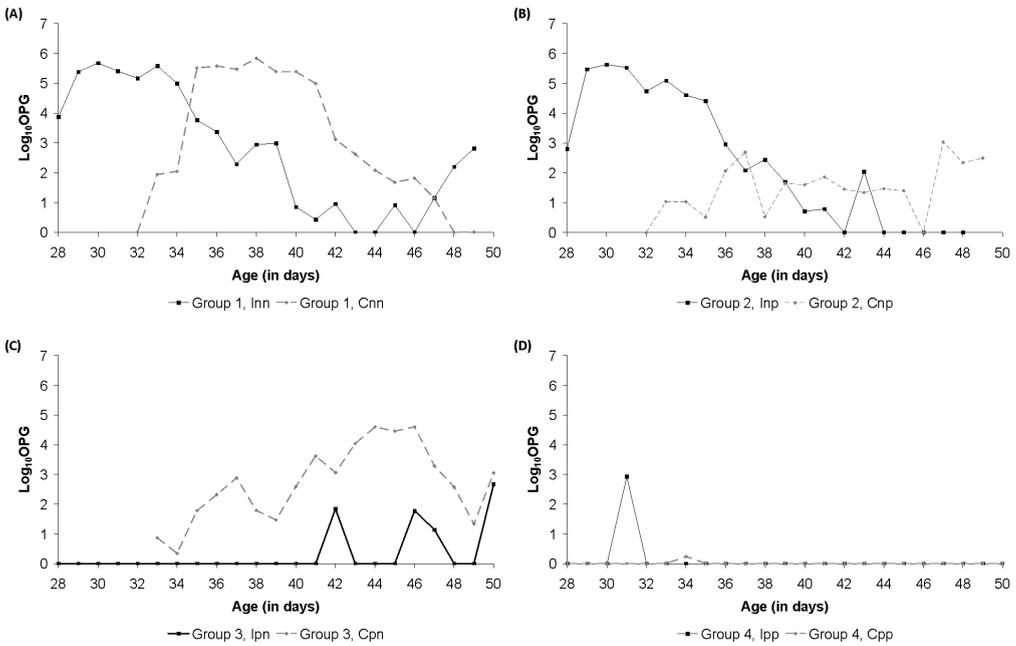
Fig. 3. Mean oocyst output for inoculated and contact birds (*n* = 6), based on McMaster oocyst counts. (A) Group 1 (*NN*), (B) Group 2 (*NP*), (C) Group 3 (*PN*), (D) Group 4 (*PP*).

Table 3
Summary of transmission characteristics: total infection delay time and total number of infected contact birds per group.

Group	Total infection delay time[a]		Total number infected *C* birds	
	McM	McM/SF	McM	McM/SF
1: *NN*	10	10	6	6
2: *NP*	35	10	5	6
3: *PN*	17	17	6	6
4: *PP*	92	92	1	1

[a] Sum of the infection delay times (based on Supplementary Tables 4-7) for all contact birds together per group.

4. Discussion

We studied the effect of heterogeneity in *E. acervulina* infection history of broilers on the acquired immunity in individual birds, and on the transmission characteristics of the parasite in the population.

4.1. Effects of infection history on immune parameters

None of the immunological cell types showed a significant sustained elevation level after infection in contact birds, and no single cell type could be linked to "protection level" or could predict oocyst output, infectivity, susceptibility or transmission between birds. In non-primed C_{NN} birds, however, a significant increase in the number of CD4$^+$ T cells was observed after contact infection, but not in primed C_{NP} chickens, despite exposure to similar amounts of oocysts. This suggests that CD4$^+$ T cells do not take part in a protective immune reaction to infection of *E. acervulina* in primed chickens, but instead rise in number during a primary infection involving many parasites, a finding that is consistent with the study of Trout & Lillehoj (1996).

CD8$^+$ T cells are known to be up-regulated after *E. acervulina* infections (Bessay et al., 1996; Swinkels et al., 2006), and are assumed to play a role in the recovery phase of infections (Lillehoj, 1988; Swinkels et al., 2007). The birds in our experiment, however, did not show a clear change in CD8$^+$ T cell kinetics. This might be due to the gradual acquisition of parasites after contact-exposure, indicating that infections established via inoculation with a high dose may differ from "natural" contact-infection after exposure. The amount of oocysts ingested might have been too low to recruit a significant amount of CD8$^+$ T cells.

In all chickens, except for the C_{NP} birds an increase in γδTCR$^+$ T cell response to an *E. acervulina* infection was observed from day 31 (I_{NN} and I_{NP} birds) and day 36 (C_{NN} birds) onwards, i.e. at the end of the endogenous phase of the life cycle in the intestinal tract (Fig. 2). Because γδTCR$^+$ T cells are associated with development of intestinal epithelia (Boismenu and Havran, 1994) and may recognise damaged tissue (Schild et al., 1994), the increase is probably a reaction to intestinal damage after *E. acervulina* infections. In inoculated birds we see that the γδTCR$^+$ T cell response remains high when the response in the non-primed contact chickens develops as well. This may indicate that the response to infection due to inoculation, the first infection cycle, is persistently high, but an alternative explanation may be that re-infection with excreted oocysts from the environment, a second infection cycle, is responsible. The latter explanation is very well possible, because oocyst excretion remains high during the second infection cycle, indicating considerable tissue damage and hence recruitment of γδTCR$^+$ T cells (Fig. 2 and 3A). Primed C_{NP} birds did not show an increase of γδTCR$^+$ T cells. It is not clear whether this suggests that a primary response to infection results in different γδTCR$^+$ T cell kinetics compared to a protective response after re-infection or that this finding can be solely explained by the reduced oocyst output and hence limited tissue damage in primed C_{NP} birds.

Although we might have identified T cell markers for either a current *E. acervulina* infection (CD4$^+$ T cells) or intestinal damage due to an *E. acervulina* infection (γδTCR$^+$ T cells), none of the single cell types showed a sustained elevated level after infection in primed or non-primed contact broilers. Therefore it can be concluded that none of the studied cell types correlates well with "protection level" nor was able to predict oocyst output, infectivity, susceptibility of previously infected birds or transmission between birds.

4.2. Effects of primary infection on oocyst output and transmission between birds

It is known that after ingestion of oocysts, the induced immune response can reduce oocyst output after re-infection (Lillehoj, 1988; Williams, 1995; Blake et al., 2005). In this experiment, the infection history reduced oocyst output in inoculated birds and contact-exposed birds. The non-primed C_{PN} birds showed a delayed peak of excretion compared to the non-primed C_{NN} birds, suggesting a delayed transmission of *Eimeria*. Although oocyst output by the primed I_{PN} birds was hardly detectable, these birds were apparently sufficiently infectious to establish contact-infection, indicating the usefulness of transmission experiments, which mimics natural infections better than experiments with "artificially" inoculated birds only. Oocyst output by the primed C_{NP} birds was significantly lower compared to that of the non-primed C_{NN} and C_{PN} birds, indicating a reduced susceptibility. However, transmission of infection did occur, which suggests that the acquired immunity was not sufficient to resist the high number of oocysts excreted by the I_{NP} bird. Whether or not this was the case for the *PP* combination cannot be determined, as it cannot be determined whether the apparent absence of oocysts in the faeces of C_{PP} birds is due to the fact that the birds were not infected or that the number was below the detection limit of the tests. Limitations of detection are demonstrated by the two C_{PN} birds and one C_{PP} bird that excreted oocysts, whereas their inoculated pen mates did not show oocyst output for the entire duration of the experiment. This suggests that small numbers of oocysts remain undetectable for both techniques. A quantitative PCR technique might be more appropriate to improve the detection limit for faecal samples with small numbers of oocysts (Blake et al., 2008; Morgan et al., 2009, Velkers et al., 2010).

Non-primed inoculated and contact birds do not differ in infection history before day 24 (inoculation), but do differ in timing and dose of ingestion of oocysts after that. Inoculated and contact birds most likely repeatedly ingested variable numbers of oocysts from the environment after day 28, but inoculated birds also received a single inoculation dose of 50,000 oocysts at day 24. Because duration of output and total output were comparable between non-primed inoculated and contact birds, the inoculation was apparently irrelevant. However, a single inoculation 18 days earlier (priming) did change the excretion pattern: priming itself caused a single high peak, and after day 24 total output and duration were significantly lower and shorter than in non-primed birds. Apparently, time intervals between oocyst ingestion are sometimes crucial for subsequent oocyst output (primed birds), and in some cases not at all (non-primed *I* and *C* birds). Only transmission experiments or experiments in which birds are allowed to re-infect themselves automatically provide a realistic timing of ingestions. It shows that simple challenge experiments, using high doses of oocysts, may give different results, and that oocyst output cannot be simply translated to predictions on outcome of infection or spread of the disease.

Another observation on timing of oocyst ingestions can be made by comparing the second infection cycles of non-primed inoculated birds (I_{NN} and I_{NP}), and of primed C_{NP} birds. These infection cycles correspond with the excretion around days 32-36 and the γδTCR[+] T cell

responses at days 36 and 39. Before the second infection cycle all these birds had experienced the same infection history with respect to numbers of oocysts (50,000 in a single dose), but they differed in the moment in life these oocysts had been given. Because the inoculated birds showed much higher oocyst excretion and elevated γδTCR$^+$ T cell levels than the primed contact birds, this suggests that inoculation at day 24 of I_{NN} and I_{NP} birds resulted in a less effective protective immune response than the priming inoculation at day 6 of C_{NP} birds. Apparently, there is a delay in the immune response, which may have considerable impact on dynamics in a larger population, and which is not yet covered by present models described by Klinkenberg and Heesterbeek (2007) and Severins et al. (2007).

In conclusion, contrary to our expectation that the measured immune parameters could indicate acquired immunity by predicting level of protection against oocyst output and transmission, no cell types were identified in this study to confirm this assumption. However, it was shown that primary infection can significantly reduce oocyst output after secondary infection. Furthermore, the natural infection of contact birds by exposure to inoculated birds has shown that undetectably low oocyst outputs can cause contact-infections, where the level of the subsequent oocyst output in contact birds depends on the level of acquired immunity obtained through the infection history of these birds. The combined study of within-host and between-host dynamics also has revealed that a delay between infection and development of immunity can occur, which can have great impact on infection dynamics in flocks.

Acknowledgements

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2010.07.005 and at the end of this manuscript.

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Supplementary data

Supplementary Table 4
Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique and infection delay time per pair for Group 1 (*NN*).

Bird[a] / Age[b]	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	Infection delay time[c] McM	McM/SF
827I_{NN}	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	++	++	-*	-*	++	--	*-	*-		-+	*-	*-		1	1
803C_{NN}					*-	+-	--	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	--	--			
764I_{NN}	-*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+-	++	-*	--	-+	*-	-+	*-		*-				2	2
756C_{NN}					*-	*-	++	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	--	-+			
311I_{NN}	+*	+*	+*	+*	+*	+*	+*	**	+*	-*	++	+*	-*	-+	-+	*-	*-	*-		*-				1	1
823C_{NN}					--	++	++	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	-*	--			
741I_{NN}	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+-	++	-*	-+	++	--	-+	-+	--	*-	*-			2	2
847C_{NN}					--	--	++	+*	+*	+*	+*	+*	+*	+*	+*	-*	-*	--	++	--	--	*-			
310I_{NN}	+*	+*	+*	+*	+*	+*		-*	--	+*	+-	+*	+*	-*	--	*-	*-	*-			*-			3	3
839C_{NN}					--	--	--	++	+*	+*	+*	+*	+*	+*	-*	+*	+*	-*	-+	+-	--				
795I_{NN}	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+-	+*	+*	+*		-+	--	++		++	++	++		1	1
857C_{NN}					*-	+-	-+	++	+*	+*	+*	+*	+*	+*	+*	-*	+*	-+	++	--	-+	-+			
Total infection delay time[d]																								10	10
Total number of infected *C* birds																								6	6

See footnotes for Supplementary Table 7.

Supplementary Table 5

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique and infection delay time per pair for Group 2 (*NP*).

Bird[a]	Age[b] 28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	Infection delay time[c] McM	McM/SF
760I_{NP}	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+-	+*	+*	+*	*+	++	*-	-+	--	--	-+			9	6
771C_{NP}					*-				--	--	*-	-+	*-	*-	++	+-	-*	*-	--	-*	*-		*-		
838I_{NP}	+*	+*	+*	+*	+*	+*	+*	+*	-*	++	++	++	-*	++	*-	+-	--	-+	--	--	*-			4	0
814C_{NP}					-+	--	-+	-+	++	++	*+	++	+*	+*	+*	+*	++	++		++	+*	+*		
853I_{NP}	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+-	++	-*	-+	-+	*-	*-							2	0
858C_{NP}					-+	--	++	-+	*-	*-	++	+-	-*	-*	*-	++	-*							
789I_{NP}	-*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+-	-*	++	--	--	++	*-							1	0
849C_{NP}					-+	++	+*	++	++	*-	-+	*-	*-	+-	-*	*-	*-							
870I_{NP}	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-+	-+	*-	--	*-	*-	*-							18	3
749C_{NP}						--	--	-+	*-	*-	*-	--	*-	*-	*-	*-	*-							
796I_{NP}	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	*-	*-	-+	--	*-	*-	*-							1	1
779C_{NP}					*-	+-	--	-+	*-	*-	*-	*-	*-	*-	*-	*-	*-							
Total infection delay time[d]																								35	10
Total number of infected *C* birds																								5	6

See footnotes for Supplementary Table 7.

Supplementary Table 6

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique and infection delay time per pair for Group 3 (*PN*).

Bird[a]	Age[b] 28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	Infection delay time[c] McM	McM/SF
797I_{PN}	*-	*-	*-	*-	*-	*-	--	*-	*-		*-		*-		*-		*-	*-		*-				1	1
301C_{PN}					*-	+-	--	+-	-*	--	--	*-	*-	++	-*	*-	++	++		++	+*	+*	+*		
318I_{PN}	*-	*-	*-	*-	*-	*-	--	*-	*-		*-		*-		*-		--	*-		*-				5	5
778C_{PN}						--	--	--	--	++	-*	--	++	+*	+*	-*	+*	+*	+*	+*	+*	+*			
821I_{PN}	*-	*-	*-	*-	*-	-+	--	*-	*-		*-		*-		*-		*-	*-	++	++	*-	-+		1	1
319C_{PN}					*-	+-	+-	--	++	+*	-*	-+	++	+*	+*	+*	+*	+*	+*	+*	+*	-+			
320I_{PN}	*-	*-	*-	*-	*-	*-	--	*-	*-		*-		*-	-*	++	*-	*-	*-		*-				3	3
866C_{PN}						--	--	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	--			
787I_{PN}	*-	*-	*-		*-	*-	--	*-	*-		*-		*-	-*	++	*-	-+	*-		--		+*		4	4
788C_{PN}					*-	--	--	--	++	+*	+*	+*	+*	+*		+*	+*	+*		+*	-+				
892I_{PN}	--	--	*-		*-	*-	--	*-	*-		*-		*-		*-		*-	*-	-+	++	*-	-*		3	3
862C_{PN}					*-	--	--	++	+*	+*	+*	-*	++	+*	+*	+*	+*	+*	+*	+*	+*	--			
Total infection delay time[d]																								17	17
Total number of infected *C* birds																								6	6

See footnotes for Supplementary Table 7.

Supplementary Table 7

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique and infection delay time per pair for Group 4 (*PP*).

Bird[a]	Age[b] 28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	Infection delay time[c] McM	McM/SF
805*I_PP*	*_	*_	*_	*_		*_	--	-+	*_		*_		*_				*_							18	18
763*C_PP*					*_	--	--		*_	*_	*_	*_	*_	*_	*_	*_	*_								
777*I_PP*	*_	--	*_	*_	*_	*_	--	-+	*_		*_		*_		*_		*_							18	18
900*C_PP*					*_	--	--	--	*_	*_	*_	*_	*_	*_	*_	*_	*_								
840*I_PP*	*_	*_	-+	++	*_	*_	--	*_	*_		*_		*_		*_		*_							18	18
743*C_PP*					*_	--	--	--	*_	*_	*_	*_	*_	*_	*_	*_	*_								
861*I_PP*	*_	*_	*_	*_	*_	-+	--	*_	*_		*_		*_		*_		*_							18	18
898*C_PP*					*_	--	--	--	*_	*_	*_	*_	*_	*_	*_	*_	*_								
831*I_PP*	--	--	*_	*_	*_	*_	--	*_	*_		*_		*_		*_		*_							2	2
331*C_PP*					*_	--	+-	--	*_	*_	*_	*_	*_	*_	*_	*_	*_								
865*I_PP*	*_	*_	-+	*_	*_	*_	--	*_	*_		*_		*_		*_		*_							18	18
781*C_PP*					*_	--	--	--	*_	*_	*_	*_	*_	*_	*_	*_	*_								
Total infection delay time[d]																								92	92
Total number of infected *C* birds																								1	1

Oocyst output data following each *I* and *C* bird: first symbol represents the results of McMaster oocyst counts (McM), second symbol shows the result of sedimentation-flotation slides (SF). +: oocysts were detected, *: not done, -: no oocysts were detected, empty cell: both tests were not done. Dark grey marked sections represent McM results above zero. Light grey marked sections represent samples that had positive SF results but had missing or negative McM values.

[a] Individual tag number followed by bird type (*I* or *C*).

[b] Age of the birds is given in days.

[c] Infection delay time = the number of days between the first day when it is considered possible for a contact bird to start excreting oocysts, i.e. at day 32 (surrounded by borders in the table) and the first day when the contact bird actually started to excrete oocysts, based solely on McM or based on McM adjusted for SF results (McM/SF = when birds were positive according to SF earlier in time than according to McM, the day with the first positive SF result was assumed to be first day of excretion).

[d] Sum of the infection delay times for all contact birds together.

Chapter 5

Oocyst output and transmission rates during successive generations of an *Eimeria acervulina* infection in broiler flocks

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Abstract - The infection dynamics of *Eimeria* species determines the clinical manifestation of the disease coccidiosis in poultry flocks, and a better understanding of the dynamics may contribute to improving control measures. Our aim was to study the course of infection and the transmission of *Eimeria acervulina* in groups of broilers by quantifying the transmission rate parameter and oocyst output. Three transmission experiments were carried out with groups of 20 male SPF broilers. At 2 days of age, one bird in each trial was orally inoculated with 5 sporulated *E. acervulina* oocysts (D0 pi). At D1 pi, the inoculated bird was housed together with 19 non-inoculated contact birds. Individual faecal droppings were examined daily from D3-D32 pi to quantify the number of oocysts per g faeces. The inoculated bird started shedding oocysts at D5 pi and contact birds between D10 and D18 pi. Oocyst output was significantly lower for first generation contact birds, i.e. birds that started excretion before D15 pi, compared to second generation contact birds, excreting from D15 pi onwards. The transmission rate parameter remained constant for both generations. These results suggest that although oocyst load increases, the transmission rate of *E. acervulina* is constant between successive generations of infection.

Keywords - *Eimeria acervulina;* broiler; transmission parameter; infection dynamics

1. Introduction

Coccidiosis, caused by several species of the protozoan parasite genus *Eimeria,* is responsible for considerable economic losses in the poultry industry, due to costs for control and poor flock performance (Williams, 1999; Shirley et al., 2005). Despite implementation of all kinds of hygienic measures, farmers have not been able to prevent infection of successive flocks (Long, 1984; Reid, 1989) and primarily rely on in-feed anticoccidials to reduce production losses. Increasing resistance against anticoccidials and public concerns about drug residues in meat, however, have stimulated demands for the development and improvement of alternative strategies, e.g. anticoccidial vaccination (Williams, 2002; Shirley et al., 2005).

The development of new control strategies requires knowledge of the biological mechanisms of *Eimeria* species infections. Most studies carried out so far focused on the interaction between *Eimeria* and the individual host (Chapman, 1999; Williams, 2001; Chapman et al., 2002; Williams, 2002; Dalloul and Lillehoj, 2005; Shirley et al., 2005). Although it provides essential knowledge, this approach excludes important elements of the processes that lead to infection and disease. Host-parasite interactions and interactions between individual hosts and their environment mutually affect each other, and can vary considerably. These interactions collectively determine the course of infection and clinical outcome for the flock (Roberts and Heesterbeek, 1995, 1998; Graat et al., 1996; Klinkenberg and Heesterbeek, 2007; Severins et al., 2007).

The course of infection is highly dependent on the transmission characteristics of the parasite (Anderson and May, 1991; Velkers et al., 2010b). Consequently, when studying effects of interventions on infection dynamics of *Eimeria* in a flock, quantitative knowledge of transmission characteristics is essential. Pair-wise transmission experiments with *Eimeria acervulina* have shown that the transmission rate parameter was independent of oocyst inoculation dose. Furthermore, contact-exposed birds shed more oocysts than inoculated birds, suggesting an increase in oocyst load with successive "generations" of infection (Velkers et al., 2010b). The effect of an increased level of contamination on transmission characteristics of *Eimeria* in groups of chickens has, however, not been demonstrated.

To study these potential variations in oocyst output between successive generations of infected chickens and to study whether these variations affect the transmission rate parameter, three transmission experiments were carried out with groups of 20 broilers. In each experiment, one bird was inoculated with 5 sporulated *E. acervulina* oocysts and the other 19 birds were contact-exposed to this one. Oocyst output was determined during 32 days and transmission rate parameters were calculated.

2. Materials and methods

2.1. Chickens and management

For each experiment, 28 male Cobb/Hybro/Ross crossbred broiler chicks were obtained from the specified pathogen free (SPF) parent flock of GD-Animal Health Service (Deventer, the Netherlands). All birds were tagged for identification at day of hatch (day 0 of age) and were housed in a floor pen with wood shavings. Room temperature was gradually decreased from 33 °C at day of hatch to 21 °C at the end of the experiment and a lighting scheme of 23 hours light per day was applied. Drinking water and a broiler starter ration (2900 kcal/kg of metabolisable energy and 21.6% crude protein) without anticoccidial drugs were offered *ad libitum*. All chickens were killed by cervical dislocation at 30, 31 and 34 days of age for experiments 1-3, respectively. The experiments were carried out according to protocols approved by the Animal Experiments Committee of Utrecht University (Utrecht, the Netherlands).

2.2. Inoculation

Five sporulated *E. acervulina* oocysts, stored at 8 °C in 2.5% (w/v) potassium dichromate, originating from a frequently rejuvenated *E. acervulina* reference laboratory strain (Weybridge W119, Central Veterinary Laboratory, Weybridge, 1990) were brought into a gelatinous capsule according to a previously described procedure (Velkers et al., 2010b). The capsule was placed in the oesophagus using a 1 ml syringe, followed by some tap water.

2.3. Experimental design

Three replicate experiments were carried out sequentially. At 2 days of age (referred to as day 0 post-inoculation, pi) one bird was inoculated with 5 sporulated *E. acervulina* oocysts and was placed in a separate floor pen. At day 1 pi, the inoculated bird (referred to as *Ino* bird) and 19 non-inoculated contact-exposed birds (referred to as *C* birds) were placed together in a 1 m^2 floor pen with wood shavings.

2.4. Detection and quantification of oocyst excretion

Single individual droppings were collected daily from day 3 pi until the end of the experiment, as described by Velkers et al. (2010a). Briefly, each chick was placed in a box for one to two hours. Faecal droppings were collected, weighed and stored at 4 °C until further processing within the same day. The number of oocysts per gram of faeces (OPG) was quantified using a modification of a McMaster (McM) oocyst counting chamber technique (Long and Rowell, 1958). The detection limit was 83 oocysts per g faeces for a faecal sample of 4 g. If no oocysts were found, a modification of the sedimentation-flotation (SF) technique (Long et al., 1976) was applied using approximately 1 g of the single dropping. The SF is considered to be more sensitive, but provides only qualitative data (Mes, 2003).

2.5. Categorisation of contact birds based on start of oocyst excretion

Based on results of previous experiments (Velkers et al., 2010b), the experimental setup was designed to allow for the occurrence of successive generations of infection. Two different generations of infection were distinguished during this experiment, based on the start of oocyst excretion. *C1* birds, "first generation contact birds", were defined as having been infected due to ingestion of oocysts excreted by the *Ino* bird (detected at day 5 pi). *C2* birds, "second generation contact birds" were assumed to have been infected by oocysts excreted by *C1* birds or by the *Ino* bird during its second excretion period. The classification was based on the observation that approximately five days passed between exposure of *C1* birds to oocysts excreted by the *Ino* bird and start of excretion by *C1* birds, i.e. from day 10 pi onwards. Similarly, it was assumed that *C2* birds could start shedding five days after the start of excretion of *C1* birds, i.e. from day 15 pi onwards. Consequently, *C1* birds were assumed to start shedding before day 15 pi and *C2* birds on or after day 15 pi. These assumptions were based on the length of the prepatent period for *E. acervulina* of 96 hours (Edgar, 1955; Joyner and Long, 1974), and sporulation time (Edgar, 1955; Norton and Chard, 1983; Graat et al., 1994).

2.6. Analysis of oocyst output data

McM oocyst counts for experiment 1 were not used for further analyses, because small numbers of oocysts were retained in the McMaster counting chambers between samples, which was not discovered until day 19 pi. Therefore, all McM test results between day 6 and day 19 pi of experiment 1 were considered unreliable.

If the SF test showed negative results, the oocyst count (OPG) was considered to be zero. Oocyst counts were log_{10}-transformed to obtain normally (Gaussian) distributed data (log_{10} (OPG+1)). The mean of the log_{10}-transformed OPG was calculated for the *Ino* and *C* birds per group per day to obtain mean oocyst excretion patterns.

The total number of excreted oocysts was estimated by calculating the area under the curve (AUC) from the daily oocyst output data for each bird using the linear trapezoidal method (Yeh and Kwan, 1978). The AUC was calculated for each bird for different periods of the experiments, i.e. days 5-9 pi, 10-14 pi, 15-19 pi, 20-24 pi and 5-28 pi and was log_{10}-transformed to normalise the data (log_{10} AUC). The first three periods contain the data of the first excretion of the *Ino*, *C1* and *C2* birds respectively; days 20-24 pi represent a period in which all *C1* and *C2* birds were actively shedding and were similar in their exposure to infectious oocysts in the litter. The period containing days 5-28 pi (TOTALOUTPUT) represents the total number of oocysts excreted during the experiment. The highest measured oocyst output during the entire experimental period (PEAK) and the day at which this occurred (DAYPEAK) were identified for each bird. Also, the highest oocyst output during the first excretion peak (FIRSTPEAK) was calculated during days 10-14 pi for *C1* birds and days 15-19 pi for *C2* birds. These variables were compared for *Ino*, *C1* and *C2* birds using one-way analysis

of variance (ANOVA), followed by Bonferroni *t*-tests to test the differences between means. Statistical significance for all analyses was set at $P < 0.05$.

Various linear regression models were evaluated to determine whether associations could be found between oocyst excretion parameters for the different types of birds. Linear mixed regression models (PROC MIXED procedure) were applied with TOTALOUTPUT, PEAK or $\log_{10}$ AUC for days 20-24 pi as dependent variables, PEAK, DAYPEAK, FIRSTPEAK and BIRDTYPE (*Ino*, *C1*, *C2*) or STARTDAY as explanatory variables and EXPERIMENT as a random factor. STARTDAY, i.e. the day of start of oocyst excretion, was entered into the models either as continuous variable or was divided into four to eight different categories. The two-tailed partial F-test (type III) was used as the elimination criterion for the variables and fit of the model was assessed by the Akaike's Information Criterion. Bonferroni adjustments for multiple comparisons were carried out to evaluate differences in least squares means. Model assumptions were evaluated by examining normality and equality of variances of the residuals. All analyses were carried out using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

2.7. Quantification of the transmission rate parameter

2.7.1. The SI-model
A stochastic susceptible-infectious model (*SI*-model) was used to describe the transmission of *E. acervulina* in these experiments. In this model it is assumed that "susceptible" birds (the naïve contact-exposed, *C* birds) can become infected through (indirect) contact with "infectious" birds (Bailey, 1975). Furthermore, it is assumed that infected birds remain infectious during the entire experimental period, because sporulated oocysts can remain infectious for the duration of the experimental period or longer (Williams, 1995). Assuming that birds have random contacts with each other independent of bird density (frequency-dependent transmission), the rate of new infections is defined as $\beta\, S\, I\, /\, N$ (de Jong et al., 1995). Here, *S* is the number of "susceptible" contact birds, *I* is the number of "infectious" birds, *N* is the total number of birds in the group and the transmission rate parameter β is the number of new infections that occurs due to (indirect) contact with one infectious animal per unit of time in a large, completely susceptible population (Anderson and May, 1991).

2.7.2. Dataset construction
For this *SI*-model a chain binomial model is assumed (Becker, 1989), i.e. during each time interval all susceptible contact birds present at the start of that interval have the same probability to become infected, $P_{inf} = 1 - exp\,(-\beta\, I\, /\, N)$. The probability for a contact bird to escape infection during time interval Δt equals $exp\,(-\beta\, I\, \Delta t\, /\, N)$ according to the Poisson distribution. Hence, the number of new infected birds (*Cases*) in an interval has a binomial distribution with the binomial total number of *S* birds at the start of the interval and probability P_{inf} as parameters, i.e. *Cases* $\sim$ *Bin (S, 1- exp* $(-\beta\, I\, \Delta t\, /\, N)$.

After uptake from the environment of infectious oocysts or after inoculation, birds were assumed to become "infectious" to other birds after a latent period of four days. The duration of this latent period is solely based on the duration of the prepatent period (Edgar, 1955; Joyner and Long, 1974). Additional time for sporulation, a process after which oocysts are infectious for other birds, was not added since previous transmission experiments (Velkers et al., 2010b) have shown that sporulation could occur within a few hours and could be considered negligible for the transmission rate calculations. Therefore, the inoculated bird was considered to be a new *Case* on day 0 pi and was assumed to be infectious, *I*, after the latent period of four days, i.e. from day 4 pi onwards. The same latent period was used to determine when birds could be considered a new *Case,* i.e. four days before start of oocyst excretion. Because faecal examinations were carried out daily, values for the number of *S* birds ($S_t = S_{t-1} - Cases_t$), *I* birds ($I_t = I_{t-1} + Cases_{t-4}$) and *Cases* could be determined from the experimental data at the start of each time interval of one day. The transmission rate estimated using the data of the three group experiments described herein is referred to as β_{group}. In addition, an alternative approach was used, where the number of *I* birds was adjusted based on the assumption that the infectivity of infected birds was spread out over four days. This assumption was considered valid, as inoculation with five oocysts generally results in excretion of oocysts over four to five days (Williams, 2001). For these calculations, each new *Case* was counted as 0.25 infectious birds after the latent period has passed, i.e. from day 4 pi onwards. For each following day, 0.25 was added until full infectivity was reached after four days, i.e. from day 7 after becoming a *Case* onwards: $I_t = I_{t-1} + 0.25\ Cases_{t-4} + 0.25\ Cases_{t-5} + 0.25\ Cases_{t-6} + 0.25\ Cases_{t-7}$. The transmission rate estimated with this alternative approach is referred to as $\beta_{groupalt}$.

2.7.3. Generalized linear regression analyses
A generalized linear model (GLM) using Stata 10.1 for Windows (StataCorp LP, College Station, USA) was applied to calculate the β with a complementary log–log link function, taking *Ln* (*I / N*) as offset variable. Maximum likelihood optimisation was applied, resulting in an intercept of this generalized regression that estimates the log β (Velthuis et al., 2003). Additional GLM models, containing either GENERATION (with value 1 for *Cases* that occurred before day 15 pi and value 2 for *Cases* from day 15 pi onwards)*,* EXPERIMENT (using dummy coding) or both as explanatory variables, were applied to determine whether the transmission rate parameters β_{group} and $\beta_{groupalt}$ were significantly different between generations and experiments. The high deviance in all GLM models indicated that the data was overdispersed. Therefore, since for the models with GENERATION and EXPERIMENT assessment of *P* values for the different variables was important, adjustments were made to correct for the overdispersion (Hardin and Hilbe, 2007). For these models, iterated reweighted least-squares (IRLS) optimisation was used, instead of the default maximum likelihood maximisation, and a scale parameter, dividing the deviance by the degrees of freedom was applied.

For three birds in experiment 1 (referred to as *U* birds, *U* = 3) the exact day when these birds became *Case*s could not be determined, as for these birds only McM results were available, which were unreliable before day 19 pi. For all other contact birds of experiment 1, the start day of excretion was determined based on SF results. Data from day 19 pi onwards showed that all three contact birds did become infected. To correct for the missing values in experiment 1, the total number of *S* birds was adjusted to 16 and the offset variable was $Ln (I / 20)$ before day 11 pi and was changed to $Ln (1 / 20 + (I - 1 / (N - U))$ from day 11 pi onwards, when most contact birds of experiment 1 became infected. For experiment 3, the data of a cross-beaked contact bird with stunted growth (*U* = 1) was omitted by changing the number of *S* birds to 18 birds and the offset variable to $Ln (1 / 20 + (I - 1 / (N - U))$ from day 11 pi onwards. The total number of birds (*N* = 20) remained unaltered for both experiments.

2.8. Simulation models

Simple stochastic simulation models were used to estimate the total number of *Cases* and the number of *C1* and *C2* birds for comparisons with the observed experimental data. These comparisons were carried out to study the effects of different approaches to transmission rate parameter estimation on the number of infected contact birds in time and to verify correct classification of *Cases* as *C1* and *C2* birds.

The same chain binomial model as used for the estimation of β was used to simulate the number of *Cases* given that we know the transmission rate parameter β and the numbers of *S*, *I* and *N* birds at the start of each interval of one day. For model I, the transmission rate parameter β_{pair} (0.70; 95% confidence interval [CI]: 0.50-0.91) estimated from previous pair-wise transmission experiments (Velkers et al., 2010b) was used. Model II used the transmission rate parameter β_{group} as estimated from the three group experiments reported herein. For model III a fictive high transmission rate parameter (β_{high}) of 8 was chosen for comparisons with the other models. For model IV it was assumed that maximum infectivity of infectious birds was reached after four days, using $\beta_{groupalt}$. In addition, the number of *I* birds was adjusted using the same method as described for the calculations of $\beta_{groupalt}$.

At the start of the simulations, 19 susceptible contact birds, *S*, and one inoculated bird were present in a total population of 20 birds (*N = 20*). The number of *Cases* at the start of each interval of one day was calculated using a binomial probability distribution with as parameters S_{t-1} (*n* = number of trials) and $1 - exp (- \beta I / N)$ (*p* = probability of "success")*.

Models were implemented in Excel (Microsoft Corporation) using the RiskBinomial function in @Risk 4.5 (Palisade Corporation, Newfield, NY, USA) with the summed total number of *Cases*, the summed number of *Cases* before day 15 (*C1*) and the summed number of *Cases* after day 15 (*C2*) as output variables. Two simulations, each consisting of 1000 iterations, were performed using Monte Carlo sampling with a randomly selected random number seed. Model fit was assessed by comparing the 95% confidence interval for the estimated total number of *Cases*, *C1* and *C2* birds with the actual observed data from the experiments.

3. Results

3.1. Oocyst excretion
In experiment 1, one contact bird died at day 13 pi, in experiment 3, one bird was euthanized at day 20 pi and three contact birds and the inoculated bird died at day 22 pi. Post-mortem examination showed that the deaths were most likely unrelated to the *E. acervulina* infection.

   The first oocyst output by the inoculated *Ino* bird was detected on day 5 pi, followed by a period of 5, 9 and 0 days without detectable oocyst output or intermittent shedding (experiments 1- 3, respectively) (Table 1 and Fig. 1). All contact birds became infected during the three experiments and started oocyst excretion between days 10-18 pi (Tables 1 and 2, Fig. 1). All contact birds of experiment 1 and 13 birds in experiments 2 and 3 were classified as *C1* birds. Six (experiment 2) and five (experiment 3) contact birds were classified as *C2* birds (Table 2). Simultaneously with the first excretion peak of *C2* birds, a second oocyst excretion peak by *C1* birds was found (Fig. 1).

   Oocyst output during days 5-28 pi and during days 20-24 pi was significantly higher for *C2* birds compared to *C1* birds (P = 0.0012 and P < 0.001 respectively); *C2* birds had a significantly higher first excretion peak (P < 0.0001) and peak output (P = 0.0021) compared to *C1* birds (Table 3). Total oocyst output and the highest oocyst peak output increased when the start of oocyst output or peak of output occurred later in the experiment.

3.2. Transmission rate parameter
The estimated transmission rate parameter β_{group}, for all experiments combined, was 3.38 (95% CI: 2.57-4.44) per day. The parameter calculated under the assumption that maximum infectivity was reached after four days resulted in a $\beta_{groupalt}$ of 5.40 (95% CI: 4.11-7.09) per day. Both estimates were neither significantly different between experiments nor between the two generations.

3.3. Simulation models
The simulation results are summarised in Fig. 2. Model I predicted that, on average, 86% of contact birds would become infected, overestimated the number of *C2* and underestimated the number of *C1* birds, and provided large 95% confidence intervals. Model III predicted that all contact birds would become infected and would be *C1* birds and provided no confidence intervals around the predictions. The predictions of model II indicated that, on average, 99% of all contact birds would become infected, of which 96% would be *C1* and 3% would be *C2* birds. Model IV predicted that none of the contact birds could escape infection and that, on average, 9% of the contact birds would be *C2* birds. Model IV was the best fitting model, as the observed experimental data fell within or most closely approached the central 95% confidence limits.

Table 1
Oocyst output data for all chickens for days 4 -19 pi of experiments 1-3.

Exp+ Bird[a]	Day pi[b] 4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1: Ino	*	+	*	−	−	−	−	+	+	*	*	*	*	*	*	**
C−1	*	−	−	−	−	−	−	−	+	*	*	*	*	*	*	+*
C−2	*	−	−	−	−	−	−	+	*	*	*	*	*	*	*	+*
C−3	*	−	*	−	−	−	−	+	+	*	*	*	*	*	*	+*
C−4	*	−	−	−	−	−	−	+	+	*	*	*	*	*	*	**
C−5	*	*	−	−	−	−	−	+	*	*	*	*	*	*	*	+*
C−6	*	−	−	−	−	−	−	+	*	*	*	*	*	*	*	+*
C−7	*	−	−	−	−	−	−	+	*	*	*	*	*	*	*	+*
C−8	*	−	−	−	−	−	−	−	*	*	*	*	*	*	*	+*
C−9	*	−	−	−	−	−	−	+	*	*	*	*	*	*	*	+*
C−10	*	*	−	−	−	−	*	+	*	*	*	*	*	*	*	+*
C−11	*	−	−	−	−	−	−	−	−	−	+	*	*	*	*	+*
C−12	*	−	−	−	−	−	−	−	*	*	*	*	*	*	*	+*
C−13	*	−	−	−	−	−	−	+	*	*	*	*	*	*	*	+*
C−14	*	*	−	−	−	−	−	+	+	*	*	*	*	*	*	+*
C−15	*	−	−	−	−	−	−	+	*	*	*	*	*	*	*	+*
C−16	*	−	−	−	−	−	−	+	*	*	*	*	*	*	*	+*
C−17	*	−	−	−	−	−	−	+	*	*	*	*	*	*	*	+*
C−18	*	−	−	−	−	−	−	+	*	*	+	*	*	*	*	+*
C−19	*	−	*	−	−	−	−	−	*	*	*	*	*	*	*	+*
2: Ino	*−	++	−*	*−	*−	−−	−−	−−	−−	*−	−−	++	+*	**	+*	+*
C−1	*−	*−	*−	*−	*−	*−	−−	++	++	+*	−+	++	+*	+*	+*	+*
C−2	*−	*−	*−	*−	*−	*−	*+	+−	−−	−−	−−	++	+*	+*	+*	+*
C−3	*−	*−	**	*−	**	*−	−−		−+		++	++	+*	+*	+*	+*
C−4	*−	*−	*−	*−	*−	*−	−−	−−	−−	−−	−−	−+	++	+*	+*	+*
C−5	*−	*−	*−	*−	*−	*−		++	++	−*	++	++	+*	+*	+*	+*
C−6	*−	*−	*−	*−	*−	*−		++	++			++	+*	+*	+*	+*
C−7	*−	*−	*−	*−	*−	*−	−−	−−	−−	−−	−−	++	+*	+*	+*	+*
C−8	*−	*−	*−	*−	*−	*−	−−	−−	−−	−−	−−	++	+*	++	+*	+*
C−9	*−	*−	*−	*−	*−	*−		++	++	−*		−+	++	+*	+*	+*
C−10	*−	*−	*−	*−	*−	*−	++	+*	++	−−	++	++	+*	+*	+*	+*
C−11	*−	**	*−	*−	*−	*−	−−	−−	*−	−−	*−	−+	++	+*	+*	+*
C−12	*−	*−	*−	*−	*−	*−	−−	−−	−−	−−		−−	++	+*	+*	+*
C−13	*−	*−	*−	*−	**	*−	−−	−−	−−	−−		++	+*	+*	+*	+*
C−14	*−	*−	*−	*−	*−	*−	*−	++	++	−−		−−	++	+*	+*	+*
C−15	*−	*−	*−	*−	**	*−	−−	−−	++	−−		++	+*	+*	+*	+*
C−16	*−	*−	*−	*−	*−	*−	++	++	++	+*	−−	++	+*	+*	**	+*
C−17	*−	*−	*−	**	*−	*−	−−	−−	++	++	−−	++	+*	**	+*	**
C−18	*−	*−	*−	*−	*−	*−	++	−−				++	+*	+*	+*	+*
C−19	*−	*−	*−	*−	**	*−	*−	++	−−	−−	++	−+	++	+*	+*	+*

Table 1 (continued)
Oocyst output data for all chickens for days 4 -19 pi of experiments 1-3.

Exp+ Bird[a]	Day pi[b]	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
3: Ino		*−	+*	−*	++	++	+*	−+	−−	++	**	++	+*	**	+*	+*	+*
C−1		*−	*−	*−	*−	*−	*−	*−	−−	++	−−	++	+*	++	+*	+*	+*
C−2		*−	*−	*−	*−	*−	*−	*−	−+	−−	*+	−*	−−	*−	++	+*	+*
C−3		*−	*−	*−	*−	*−	*−	*−	−−	*+	++	++	+*	+*	+*	+*	+*
C−4		*−	*−	*−	*−	*−	*−	*−	−−	−−	−−	−+	*−	++	++	+*	+*
C−5		*−	*−	*−	*−	*−	*−	*−	−−	−−	−−	++	−+	++	+*	+*	+*
C−6		*−	*−	*−	*−	*−	*−	−−	−−	−−	−−	−+	*−	*−	*−	++	+*
C−7		*−	*−	*−	*−	*−	*−	−−	*−	−−	−−	++	+*	+*	+*	+*	+*
C−8		*−	*−	*−	*−	*−	*−	*−	−+	*−	*+	++	−−	*−	++	+*	+*
C−9		*−	*−	*−	*−	*−	*−	*−	−−	−−	−−	−−	*−	*−	++	+*	+*
C−10		*−	*−	*−	*−	*−	**	*−	−−	*−	−−	++	−−	−−	+*	+*	+*
C−11		*−	*−	*−	*−	*−	*−	*−	−−	++	−−	−−	*−	*−	−+	++	+*
C−12		*−	*−	*−	*−	*−	*−	*−	−−	−−	*−	*−	*−	*−	++	++	+*
C−13		*−	*−	*−	*−	*−	*−	*−	−−	−−	−−	−−	++	*−	++	+*	+*
C−14		*−	*−	*−	*−	*−	*−	*−	−−	−−	++	++	+*	−−	++	+*	+*
C−15		*−	*−	*−	*−	*−	*−	*−	−−	−−	−−	−−	++	+*	+*	+*	+*
C−16		*−	*−	*−	*−	*−	*−	*−	++	++	++	++	+*	+*	+*	+*	+*
C−17		*−	*−	*−	*−	*−	*−	*−	−−	−+	++	++	+*	+*	+*	+*	+*
C−18		*−	*−	*−	*−	*−	**	*−	−−	−−	−+	−−	*−	++	++	+*	+*

The first symbol shows results of McMaster oocyst counts (McM), the second symbol of sedimentation-flotation slides (SF). Positive samples according to McM, SF or both tests are marked grey: + Oocysts detected; * test not done; − no oocysts detected.
[a] The results are shown for the inoculated (*Ino*) and for 19 contact birds (*C*) of experiments 1-3. The number of the experiment precedes the notation for the *Ino* bird and is underlined. The data of one cross-beaked *C* bird was excluded for experiment 3 and the McM results for experiment 1 are not shown, as these were unreliable until day 19 pi.
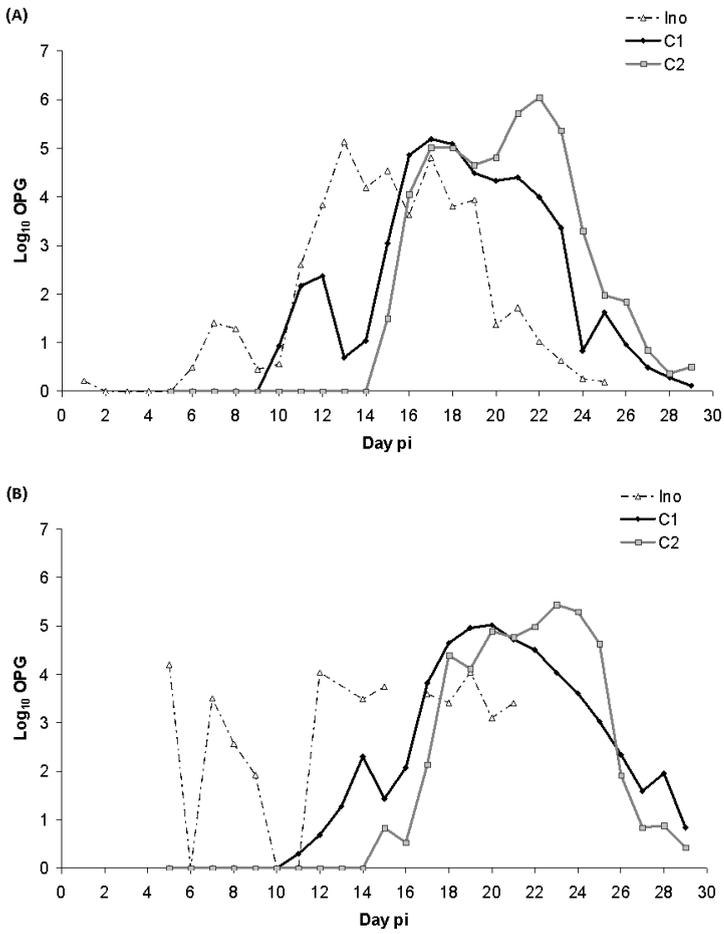[b] Day pi = days after inoculation of the *Ino* bird (day 0 pi = day 2 of age).

Fig. 1. Mean oocyst excretion (mean of $\log_{10}$ (OPG+1)) for *Ino*, *C1* and *C2* birds per day for experiments 2 (A) and 3 (B).

Table 2
The number of contact birds starting with oocyst excretion per day pi for experiments 1-3.

Exp	Test	Day pi[a] 10	11	12	13	14	15	16	17	18	Number of contact birds with known day of start of output / Total number of infected contact birds[b]
1	SF	0	14	1	0	1	0	0	0	0	16 / 19
	McM[c]										
2	SF	4	6	3	0	0	5	1	0	0	19 / 19
	McM	3	7	2	0	1	3	3	0	0	19 / 19
3	SF	0	3	4	2	4	3	0	2	0	18 / 19
	McM	0	1	2	3	4	2	2	3	1	18 / 19

Results are based on McMaster counts (McM) or sedimentation-flotation slides (SF). The inoculated bird (*Ino*) was positive at day 5 pi in each of the experiments (results not shown in table). Note that for calculations of the transmission rate parameter contact birds (*C*) were considered to be a new *Case* four days prior to the start of oocyst excretion.

[a] Day pi = days after inoculation of the *Ino* bird (day 0 pi = day 2 of age).

[b] The number of newly excreting *C* birds per day pi represents only *C* birds for which the day of excretion could be accurately determined (i.e. excluding three *C* birds for experiment 1 and one for experiment 3). In all three experiments, all 19 *C* birds showed oocyst excretion at some point during the experiment.

[c] For experiment 1 McM counts were not reliable until day 19 pi and were not used to determine day of start of excretion.

Table 3
Total oocyst output for different periods and during the first and highest peak output for the inoculated (*Ino*) bird and first generation (*C1*) and second generation contact birds (*C2*) for experiments 1, 2 and 3.

Bird Type[d]	Oocyst output[e] Days 20-24 pi $\log_{10}$ AUC (95% CI)	Total oocyst output[e] Days 5-28 pi $\log_{10}$ AUC (95% CI)	First peak of oocyst output[e] $\log_{10}$ OPG (95% CI)	Highest peak output[e] $\log_{10}$ OPG (95% CI)
Ino	4.16 (3.88-4.43)[a]	5.55[a]	4.16 (4.06-4.25)[ab]	5.22[a]
C1	5.17 (4.96-5.37)[b]	5.82 (5.68-5.95)[a]	2.98 (2.47-3.49)[a]	5.37 (5.21-5.52)[a]
C2	6.18 (5.96-6.39)[c]	6.29 (6.09-6.50)[b]	5.02 (4.85-5.19)[b]	5.92 (5.67-6.17)[b]

The data represent the means and 95% confidence intervals (95% CI), based on the normal distribution.

[a-c] Values within the same column with different superscripts differ significantly ($P < 0.05$) based on one-way ANOVA analysis, followed by Bonferroni *t*-tests.

[d] Results are shown for the inoculated (*Ino*) bird and first generation (*C1*) and second generation contact birds (*C2*).

[e] $\log_{10}$ AUC of oocyst output was calculated from daily OPG values per bird for days 20-24 pi, when all birds were actively shedding oocysts, and as a measure for total oocyst output during the experiment, i.e. for days 5-28 pi. The first peak output represents the highest $\log_{10}$ OPG value for days 10-14 pi for *C1* birds and days 15-19 pi for *C2* birds. The highest peak output represents the highest measured $\log_{10}$ OPG during the entire experiment. For calculation of the total and highest peak output, all data of birds that died during the experiment and for birds of experiment 1, with unreliable oocyst counts until day 19 pi, were excluded. For these variables, only the data for the *Ino* bird of experiment 2 is shown.
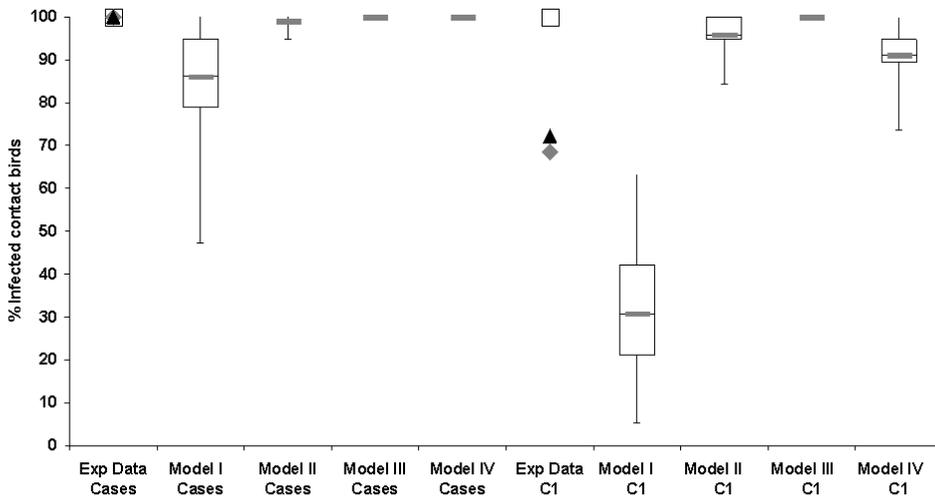
Fig. 2. The percentage of infected contact birds for experiments 1 (□), 2 (♦) and 3 (▲) and as simulated by four different models. The total number of infected contact birds (*Cases*) and number of first generation contact birds (*C1)* are expressed as a percentage of the total number of contact birds used for the analyses, i.e. 19 for simulation models I-IV and 16, 19 and 18 birds for experiments 1, 2 and 3 respectively. Results of the simulations are summarised in customised box and whiskers plots, with the lower and upper outline of the box representing the 25% and 75% percentile and the horizontal grey line (—) the mean. The whiskers correspond to the lower and upper bounds of the 95% confidence interval. Four different transmission rate parameter estimations were used as input for the simulation models, i.e. β_{pair} from previous pair-wise experiments (Model I), β_{group} from the three group experiments (Model II), β_{high}, a hypothetical high transmission rate (Model III) and $\beta_{groupalt}$, an adjusted transmission rate estimation from the group experiment data, based on the assumption that infectivity was spread out over four days (Model IV).

4. Discussion

The aim of this study was to study infection dynamics of *E. acervulina* in groups of broilers and to determine whether oocyst output and transmission changed during the course of infection. Pair-wise transmission experiments have shown that contact-infected birds excreted more oocysts than inoculated birds, but that transmission rates were independent of oocyst inoculation dose (Velkers et al., 2010b). An increase in oocyst load during progression of a flock infection was also reported for field experiments, where oocyst load and occurrence of clinical signs showed a peak at four to five weeks of age (Graat et al., 1996; Williams, 2002). Consequently, oocyst load was expected to increase with successive generations of infection, whereas the transmission rate was expected to remain constant. Also in our experiments, the oocyst output by infected birds increased when they became infected later during the course of infection. All contact birds became infected and the transmission rate parameter was not significantly different between the two successive generations of infection. These results suggest that, not the oocyst load, but perhaps the

probability of birds to come into contact with infectious faecal contamination of the environment, determines transmission.

The transmission rate parameter for the group experiments was 3.4 day^{-1}, which means that one infectious bird can infect on average 3.4 susceptible birds in one day. This transmission rate parameter estimate was nearly five times higher than the one based on the pair-wise experiments (0.7 day^{-1}) (Velkers et al., 2010b). This result was unexpected, and therefore, simulations were done to study potential effects of different approaches to transmission rate estimation on the number of infected contact birds in time. The simulations with model I illustrated that a transmission rate of 0.7 day^{-1} could not explain the dynamics observed in the groups, because this model predicted that not all 19 contact birds in a group would become infected. Furthermore, the predicted proportion of second generation birds was much higher than observed in the experiments. Also according to model II, using the transmission rate parameter of 3.4 day^{-1}, not all birds would become infected, whereas in all three group experiments none of the contact birds escaped infection. Furthermore, this model underestimated the number of second generation birds. When an even higher transmission rate of 8 day^{-1} was used in simulation model III, it was predicted that all contact birds would become infected, without the presence, however, of second generation birds, which was not consistent with the observations in two of the experiments.

The simulation model that showed the best fit with the observed data was model IV. According to predictions by this model, all contact birds would become infected and furthermore, both first and second generation birds could be present. For the calculations used for this model, it was assumed that infectivity builds up over four days, after which maximum infectivity is reached. This assumed build up of infectivity was based on the hypothesis that the probability of infection of contact birds might increase when, in time, an increasing number of patches of contaminated faeces become available in the pen. The oocysts excreted in preceding days can survive in the contaminated litter, but may become unavailable for ingestion after a few days, possibly due to subsiding to lower levels of the litter or disintegration due to ammonia and bacteria. Therefore, it is assumed that some time will pass before the increase in infected litter surface matches the decrease, after which the infected area remains constant. Consequently, especially at the start of a flock infection when only a few chickens are excreting contaminated faeces, the transmission rate most likely gradually increases to a plateau level, at which it remains constant for as long as the number of infectious individuals remains constant. In the pair-wise experiments, only one contact bird was present, and therefore only one contact infection could take place. Therefore, the estimate for the transmission rate reflected the pre-plateau infectivity, whereas in the group additional infections could occur after the first infections, reflecting the higher plateau infectivity. Consequently, the transmission rate parameter estimated from the pair-wise experiments probably provided an underestimation of this parameter for a group. For future transmission experiments with *Eimeria* and perhaps also for other pathogens with similar infection dynamics, the mathematical models used for the estimation of the transmission rate should correct for the effects of this build up phase.

Our study shows that oocyst load in the environment and the time at which birds become infected influence oocyst output in a flock, but has no significant effect on the transmission rate parameter. Furthermore, the results imply that the distribution of infectious faecal material in the environment, and therefore factors that can influence this process, such as bird movements through the poultry house, may affect transmission of *Eimeria* species in a flock. Therefore, the influence of such factors on infection dynamics and efficacy of control measures should be taken into account in future studies.

Acknowledgements

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Chapter 6

Transmission of a live *Eimeria acervulina* vaccine and response to infection in vaccinated and contact-vaccinated broilers

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Abstract - Live vaccines for coccidiosis control are infrequently used in broilers, mainly due to doubts about efficacy and cost-effectiveness of vaccination compared to anticoccidial drugs. More insight in transmission of vaccine and wild-type strains can facilitate optimisation of vaccination strategies and might increase its use as an alternative for anticoccidials. The aim of this study was to quantify transmission of a live *Eimeria acervulina* vaccine strain and to determine the degree of protection against a subsequent infection with a wild-type *E. acervulina* strain. An experiment was carried out with four groups of 22 SPF broilers. At 2 days of age, 11 birds of groups 2-4 were vaccinated directly by oral application of *E. acervulina* oocysts of the Paracox vaccine and 11 birds were placed in contact with these birds (contact-vaccinated). Birds in group 1 remained unvaccinated (controls) and were not exposed to vaccinated birds. At day 28 of age, six groups of 10 birds were formed with two groups (duplo) for each treatment group, i.e. vaccinated, contact-vaccinated or unvaccinated control birds. Five birds of each group were orally inoculated with wild-type *E. acervulina* oocysts and five were contact-exposed. Single droppings were examined daily from days 5-49 of age for oocyst output and to determine the time of infection. The transmission rate of the vaccine strain was estimated to be 1.6 per day and of the wild-type strain 2.3, 8.7 and 17.6 per day for vaccinated, contact-vaccinated and unvaccinated birds, respectively. Although transmission of wild-type coccidia was not significantly reduced in vaccinated or contact-vaccinated groups, both groups were equally protected against high oocyst output after infection compared to unvaccinated groups.

Keywords - *Eimeria acervulina*; vaccination; broiler; transmission; oocyst output; infection dynamics

1. Introduction

Coccidiosis is an economically important disease in chickens, caused by species of the protozoan parasite genus *Eimeria*. Increasing resistance against anticoccidial drugs and public concerns and legislative bans against medicated feed have increased demands for alternative control strategies. The parasite is highly immunogenic and an infection may rapidly result in a protective immune response. Vaccination may therefore be a good alternative for anticoccidial drugs to protect against symptoms or production losses and is rather successfully applied worldwide in layer and breeder flocks (Allen and Fetterer, 2002; Shirley et al., 2005). Up to now, however, the use in broiler flocks is limited, most likely due to relatively high costs in relation to the small profit margins, doubts about the timely onset of protective immunity in birds with such a short life span and concerns about adverse effects on growth (Williams, 2002; Shirley et al., 2005). Improved efficacy of vaccination strategies could be helpful to increase its use as an alternative for anticoccidials.

The short life of broilers makes it challenging to induce sufficient and timely protection of a large part of the flock to protect against infections with circulating wild-type strains. Many authors have suggested that a crucial factor for efficacy of vaccination is the uniformity of uptake of the appropriate oocyst dose by a large proportion of birds in the flock at an early age. It is assumed that this is often not fully achieved, probably due to the mass application of live vaccines in broiler flocks (Chapman et al., 2002; Williams, 2002; Chapman et al., 2005; Shirley et al., 2005). Another crucial factor is the immunity boosting effect of multiple re-infections with oocysts from the vaccine or wild-type strains from the environment. Both factors are influenced by the transmission characteristics of vaccine or wild-type strains in the flock. More knowledge of transmission of these *Eimeria* strains in a flock is therefore essential to improve efficacy of vaccination strategies.

In most studies on vaccine efficacy in broilers published to this date, body weight gain, feed conversion, intestinal lesions and oocyst shedding were determined to evaluate protection after challenge-inoculation or after finishing the production cycle (Williams, 1992; Danforth, 1998; Williams et al., 2000; Chapman et al., 2002; Crouch et al., 2003; Chapman et al., 2005; Shirley et al., 2005). One of the questions that remained unanswered in these studies is, however, if and when birds that do not ingest the initially applied vaccine oocysts, do ingest oocysts excreted by vaccinated birds and whether this "contact-vaccination" induces an adequate immune response that can induce sufficient and timely protection against (the adverse effects of) a subsequent infection with a wild-type strain.

Neither the transmission characteristics of vaccine strains, nor the efficacy of "contact"-vaccination, are currently known. Therefore, a transmission experiment was carried out to quantify transmission of an *Eimeria acervulina* vaccine strain between birds. Furthermore, transmission and oocyst output after an infection with a wild-type *E. acervulina* strain were compared for unvaccinated, vaccinated and contact-vaccinated birds.

2. Materials and methods

2.1. Chickens and management

A total of 100 male Cobb/Hybro/Ross crossbred broiler chickens were obtained from the specified pathogen free parent flock of GD-Animal Health Service (Deventer, the Netherlands). All birds were tagged for identification at day of hatch and were divided over two floor pens with wood shavings. At day 2 of age, the birds were randomly divided into four groups (groups 1-4), each consisting of 22 birds. Groups 2, 3, and 4 were placed in the same experimental unit (stable A). Group 1 was placed in a separate experimental unit (stable B). Each group was housed in a cage with a surface of 1.2 m^2, containing 1 kg/m^2 of wood shavings. Birds in groups 2-4 were vaccinated according to the protocol described below (section 2.2.). The birds in group 1 remained unvaccinated. From day 28 of age onwards, six groups (1a, 1b, 2a, 2b, 3a and 3b) consisting of 10 chickens were formed. Each group was housed in stable A in a cage of 0.6 m^2 with 1 kg/m^2 of clean wood shavings. The treatments for each group are described below (section 2.3.). Room temperature was gradually decreased from 36 °C at day of hatch to 21 °C at the end of the experiment and a lighting scheme of 23 hours light per day was applied, with alternating periods of white and red light. Drinking water and a broiler starter ration (2900 kcal/kg of metabolisable energy and 21.6% crude protein) without anticoccidial drugs were offered *ad libitum*. All chickens were killed at the end of the experiment at 49 days of age by cervical dislocation. The experiment was carried out according to protocols approved by the Animal Experiments Committee of Utrecht University (Utrecht, the Netherlands), in accordance with the Dutch Experiments on Animals Act.

2.2. E. acervulina vaccine and challenge inocula

A suspension of sporulated oocysts of a live attenuated precocious *E. acervulina* line of the Paracox vaccine (Williams, 1992), kindly provided by Intervet/Schering-Plough Animal Health (Boxmeer, the Netherlands) was diluted with tap water and suspended in a 1:1 dilution of a 30% sucrose solution. Oocyst number and sporulation percentage was determined microscopically using a Fuchs-Rosenthal haemocytometer counting chamber. The vaccine was administered by oral inoculation of 600 sporulated *E. acervulina* oocysts in a volume of 0.42 ml, using a 1 ml syringe with a blunt crop needle. The challenge inoculum, consisting of 5000 sporulated *E. acervulina* oocysts (Weybridge W119 strain), kindly provided by GD-Animal Health Service (Deventer, the Netherlands), was prepared and administered in a volume of 1.10 ml according to the same procedures as described for the vaccine.

2.3. Experimental design

The experiment consisted of a vaccination period, from days 2-27 of age and a challenge period, from days 28-49. Groups, number of birds, treatments and measurements during the

vaccination and challenge period are shown in Table 1 and are summarised in a flow chart in Fig. 1.

At the start of the vaccination period, at day 2 of age and day 0 post-vaccination (day 0 pv), 88 birds were randomly divided into four groups, each consisting of 22 birds. In groups 2-4, 11 randomly chosen birds were vaccinated ("vaccinated", V birds) with 600 sporulated *E. acervulina* oocysts of the Paracox vaccine and 11 other birds ("contact-vaccinated", CV birds) were sham-vaccinated with water. At day 1 pv, CV birds were placed in the same cage with the V birds. All 22 birds of group 1 were sham-vaccinated with water ("non-vaccinated", NV birds) and were housed in a different stable (stable B) to prevent cross-contamination with vaccinal oocysts.

At the start of the challenge period, at day 28 of age and day 0 post-challenge (day 0 pc), birds from groups 1-4 were divided into six groups of 10 birds. Groups 1a and 1b consisted of NV birds, groups 2a and 3a of V birds and groups 2b and 3b of CV birds. Five birds per group were challenged ("inoculated", Ino birds) with 5000 sporulated oocysts of a wild-type strain of *E. acervulina* and five were not ("contact-exposed" to the challenge strain, CC birds). The CC birds were placed in the same cage with Ino birds from day 1 pc onwards.

2.4. Detection and quantification of oocyst excretion

Quantification of oocyst excretion and evaluation of the infection status of individual birds for estimation of the transmission rate parameter was determined by collecting and examining individual faecal droppings. During the vaccination period, single individual droppings were collected daily in groups 2-4 from day 5 of age (day 3 pv) for V birds and day 7 of age for CV birds onwards. Pooled fresh faecal samples were collected from the litter of group 1 (NV birds) in stable B on days 10, 17 and 25 of age and for each bird individually on day 28 to confirm absence of oocyst excretion. During the challenge period, all birds were sampled daily from day 31 of age (day 3 pc) for Ino birds and from day 35 of age onwards for CC birds until the end of the experiment at day 49. When V, CV, Ino or CC birds had been shedding oocysts for at least seven days, sampling was carried out with intervals of one or two days.

The single droppings were collected and analysed according to procedures described by Velkers et al. (2010a). Briefly, each chick was placed in a cardboard box with a clean paper sheet for approximately two hours. The single dropping was weighed and the number of oocysts per g of faeces (OPG) was determined, according to a modification of a McMaster oocyst counting chamber technique (McM) described by Long and Rowell (1958). The detection limit was 83 oocysts per g faeces for a faecal sample of 4 g. If no oocysts were found, a modification of the sedimentation-flotation (SF) technique (Long et al., 1976) was applied using approximately 1 g of the single dropping. The SF is considered to be more sensitive, but provides only qualitative data (Mes, 2003). If the SF test showed negative results and McM data were not available, the OPG was considered to be zero.

Table 1
Outline of the experimental groups, treatments and measurements during the vaccination and challenge period.

Group[a]	Vaccination[b]	Measurements[c]	Challenge / wild-type inoculation[d]	Measurements[c]
1: Non-vaccinated Challenged	11 birds non-vaccinated (NV) 11 birds non-vaccinated (NV)			
1a			5 NV-birds challenged (NV1-Ino) 5 NV-birds non-challenged (NV1-CC)	OPG + transmission wild-type strain
1b			5 NV-birds challenged (NV1-Ino) 5 NV-birds non-challenged (NV1-CC)	OPG + transmission wild-type strain
2: Vaccinated Challenged	11 birds vaccinated (V) 11 birds non-vaccinated (CV)	OPG + transmission vaccine		
2a			5 V-birds challenged (V2-Ino) 5 V-birds non-challenged (V2-CC)	OPG + transmission wild-type strain
2b			5 CV-birds challenged (CV2-Ino) 5 CV-birds non-challenged (CV2-CC)	OPG + transmission wild-type strain
3: Vaccinated Challenged	11 birds vaccinated (V) 11 birds non-vaccinated (CV)	OPG + transmission vaccine		
3a			5 V-birds challenged (V3-Ino) 5 V-birds non-challenged (V3-CC)	OPG + transmission wild-type strain
3b			5 CV-birds challenged (CV3-Ino) 5 CV-birds non-challenged (CV3-CC)	OPG + transmission wild-type strain
4: Vaccinated Non-challenged	11 birds vaccinated (V) 11 birds non-vaccinated (CV)	OPG + transmission vaccine		

[a] Groups were referred to as groups 1-4 during the vaccination period (aligned left) and as groups 1a, 1b, 2a, 2b, 3a and 3b during the challenge period (aligned right).
[b] Oral inoculation with 600 sporulated *E. acervulina* oocysts from the Paracox vaccine at day 2 of age (day 0 pv).
[c] Individual faecal samples were examined daily to determine the presence and number of oocysts per g of faeces (OPG) using the sedimentation-flotation and McMaster oocyst counting technique respectively. These measurements were used to determine infection status of birds for transmission rate calculations and for oocyst output quantification.
[d] Oral inoculation with 5000 sporulated *E. acervulina* oocysts of a wild-type (Weybridge) strain at day 28 of age (day 0 pc).
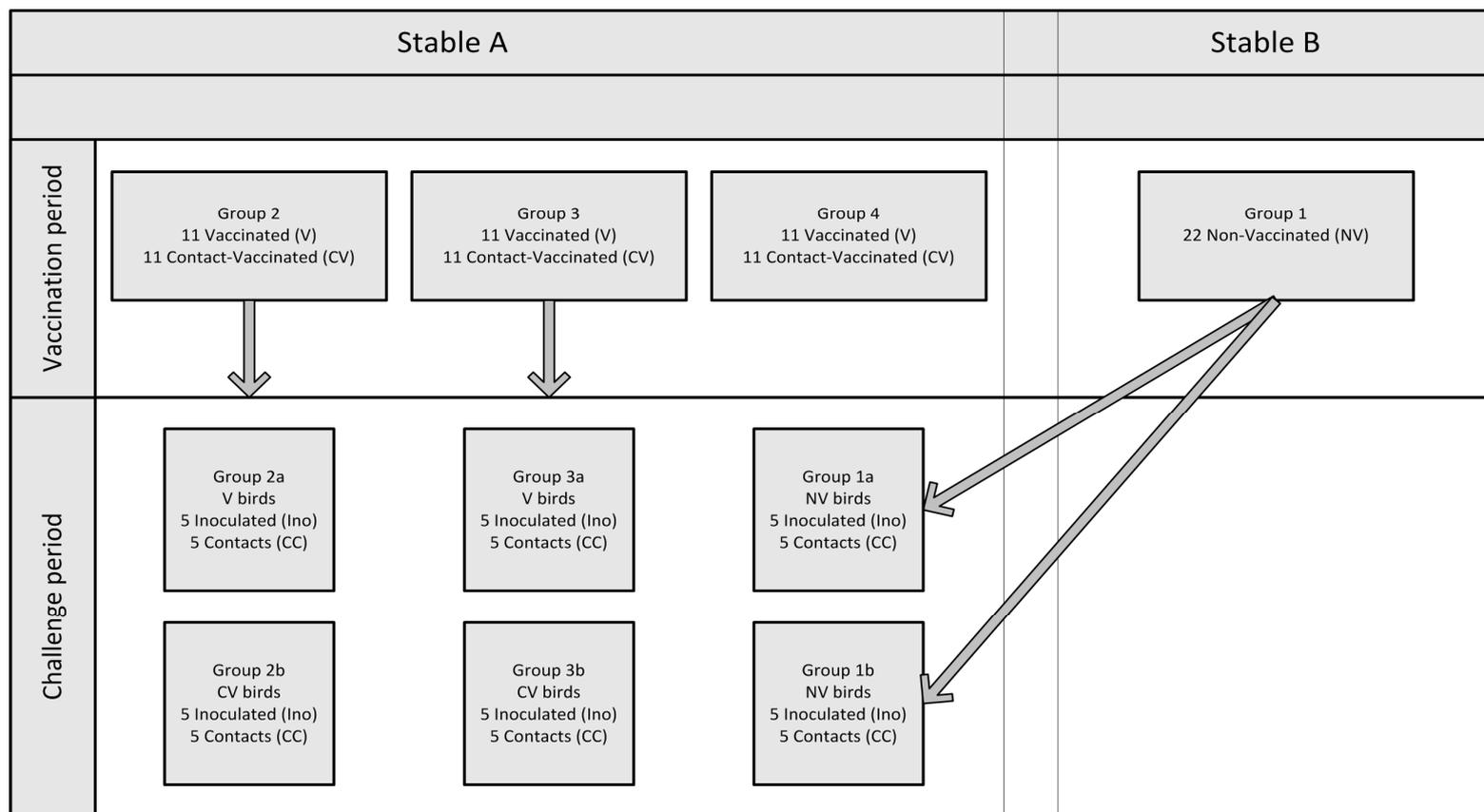
Fig. 1. Flow chart of the experimental design illustrating the group composition during the vaccination and challenge period of the experiment. At the start of the vaccination period (day 2-27 of age), 11 birds of Groups 2-4 were vaccinated (V birds) with 600 *E. acervulina* vaccine oocysts and 11 birds were not vaccinated (CV). Birds of group 1 remained unvaccinated (NV) and were kept in stable B. During the challenge period (day 28-49 of age), six groups of 10 birds were housed in stable A: groups 1a and 1b (NV), 2a and 3a (V) and 2b and 3b (CV). Five birds per group were challenged (inoculated, Ino birds) with 5000 sporulated oocysts of a field strain of *E. acervulina* at day 28 of age and five were not (contact-challenged, CC birds).

2.5. Statistical analyses of oocyst excretion data

Oocyst counts were $\log_{10}$-transformed to obtain normally (Gaussian) distributed data ($\log_{10}$ (OPG+1)) and the mean of the $\log_{10}$-transformed (OPG +1) was used to obtain oocyst excretion patterns per day for the different groups. As a measure for the total number of excreted oocysts, the area under the curve (AUC) was calculated from the daily non-transformed oocyst output data for each bird using the linear trapezoidal method (Yeh and Kwan, 1978). AUC was subsequently $\log_{10}$-transformed to obtain normally distributed data and the mean $\log_{10}$-transformed AUC ($\log_{10}$ AUC) was calculated for V, CV and NV birds and for Ino and CC birds for different periods of the experiment. For the vaccination period, the mean $\log_{10}$ AUC was determined for the first peak of oocyst output of V birds (days 3-7 pv) and CV birds (days 7-11 pv) and for the entire vaccination period (days 3-23 pv). For comparisons of output of V and CV birds for an excretion period of equal duration, $\log_{10}$ AUC was determined for days 7-23 pv, i.e. starting after the first (inoculation-induced) peak of excretion by V birds ("total corrected output"). The mean $\log_{10}$ AUC was determined for days 3-17 pc for Ino birds and for days 7-21 pc for CC birds.

Linear mixed regression models (PROC MIXED procedure) were used to determine associations between the $\log_{10}$ AUC data for different periods as dependent variable, and *vaccination status* (V or CV) for the vaccination period and *vaccination status* (V, CV or NV) and *challenge status* (Ino, CC) for the challenge period as explanatory variables. *Cage* was added as a random effect and interaction terms were added if significant. For comparisons between groups, linear regression models (PROC GLM procedure) were applied. The $\log_{10}$ AUC data for the different periods served as dependent variable and *vaccination status* (V, CV), *group*, *challenge status* (Ino, CC) and interaction terms, if significant, were added as explanatory variables. Statistical analyses were performed in the software package SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) and statistical significance was set at $P < 0.05$. The two-tailed partial F-test (type III) was used as the elimination criterion for the variables during model building and fit of the model was assessed using the Akaike's Information Criterion (AIC) for linear mixed models and the R^2 for general linear models. Bonferroni adjustments for multiple comparisons were carried out to evaluate differences in least squares means. Model assumptions were evaluated by examining normality and equality of variances of the residuals.

2.6. Quantification of transmission

A stochastic susceptible-infectious model (*SI*-model) was used to describe the transmission of the *E. acervulina* vaccine and challenge strains in these experiments. In this model it is assumed that "susceptible" birds (*S*), i.e. non-inoculated birds (CV or CC birds), can become infected after ingestion of oocysts shed by infectious (*I*) birds (Bailey, 1975) and remain infectious during the entire experimental period, as sporulated oocysts can remain infectious for the duration of the experimental period or longer (Williams, 1995). Assuming that birds have random contacts with each other, independent of bird density (frequency-dependent

transmission), the rate of new infections is defined as $\beta\, S\, I\, /\, N$ (de Jong et al., 1995). Here, N is the total number of birds in the group and the transmission rate parameter β is the number of new infections that occurs due to contact with one infectious animal per unit of time in a large, completely susceptible population (Anderson and May, 1991). The number of new infected birds ("*Cases*") in an interval has a binomial distribution with the binomial total number of S birds at the start of the interval and the probability for S birds to become infected as parameters (de Jong et al., 1995): *Cases* ~ *Bin (S, 1- exp (-β I Δt / N)*.
Based on the daily individual OPG and SF results, the start of excretion could be determined for each bird and datasets were created with the number of new *Cases*, S, I and N birds for each day of the experiment for the vaccination and challenge period.

After uptake of oocysts, birds were assumed to become infectious after a latent period of three days, and, similarly, birds were considered as a new *Case* three days before the start of oocyst excretion. According to literature, the prepatent period for the precocious *E. acervulina* vaccine strain from the Paracox vaccine is approximately 62 (Shirley and Bedrník, 1997) and 96 hours for wild-type *E. acervulina* strains (Edgar, 1955; Joyner and Long, 1974; McDougald, 2008). Because the prepatent periods for the vaccination and challenge strain, as deduced from the experimental data, differed less than the sampling interval of one day, the same latent period of three days was used for the vaccination and challenge period, to avoid numerical problems with the datasets in the statistical model. Additional time for sporulation was not included in the latent period, as sporulation could be considered negligible for the transmission rate calculations according to previous experiments (Velkers et al., 2010b). For each of three observations where five *Cases* and five S birds were present at the same day (groups 1a, 1b and 3a) the number of *Cases* was adjusted to 4.99. This only slightly reduced the estimation of the transmission rate parameter for the NV group, but solved numerical problems with extremely large standard errors and 95% Confidence Intervals (CI).

A generalized linear model (PROC GENMOD) using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was applied to calculate the β with a complementary log–log link function, taking $Ln\ (I\,/\,N)$ as offset variable. Maximum likelihood optimisation was applied, resulting in an intercept of this generalized regression that estimates the log β (Velthuis et al., 2003; Bos et al., 2009). The scaled deviance per degree of freedom was forced to one. Bonferroni corrections were applied for multiple comparisons of the transmission rate parameter between V and CV birds, V and NV birds and CV and NV birds.

3. Results

3.1. Oocyst excretion

During the vaccination period, V birds started shedding oocysts from days 3-4 pv onwards. All CV birds started excreting from days 6-9 pv onwards (Supplementary Tables 1-3, Fig. 2). V birds had a second oocyst excretion peak coinciding with the first shedding of CV birds.

The peak output of CV birds and the total oocyst output for days 7-23 pv was significantly higher for CV than for V birds (Table 2). For V birds, the first peak of excretion was not significantly different between groups. CV birds of group 4 shed significantly lower numbers of oocysts during the first peak than CV birds of groups 2 and 3 (Table 2).
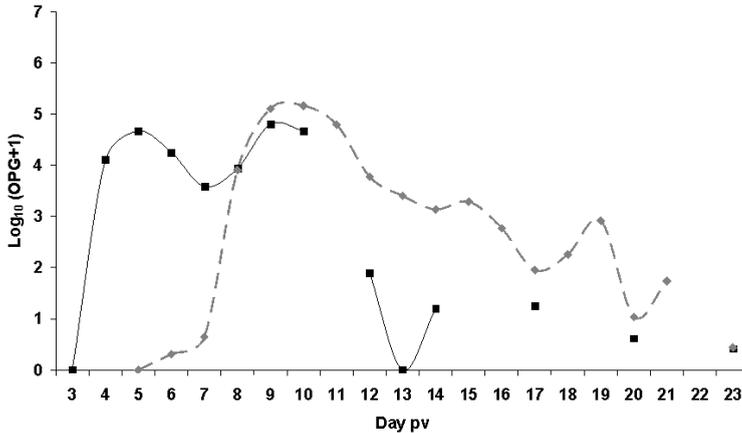

Fig. 2. Mean oocyst excretion in $\log_{10}$ (OPG+1) by vaccinated (—■—) and contact-vaccinated (—♦—) birds during the vaccination period, for groups 2, 3 and 4 combined. Vaccinated birds were inoculated with the vaccine at day 0 pv.

After challenge, Ino birds started shedding from day 4-6 pc. All CC birds excreted oocysts from days 7-11 pc onwards (Supplementary Tables 4-6, Fig. 3). The first peak of oocyst output occurred on day 5 pc for NV, and day 6 pc for V and CV birds. A second peak of output, around the start of shedding of the CC birds, occurred on day 10 pc for NV birds, and day 13 pc for V and CV birds. Total oocyst output during the challenge period did not differ significantly between CC and Ino birds (Table 2). The total oocyst output during the challenge period was significantly higher for NV birds than for CV and V birds, but this variable was not significantly different between CV and V birds (Table 2 and Fig. 4).

3.2. Transmission of vaccine and challenge strains
The parameter β for the vaccine strain was estimated to be 1.58 day^{-1} (95% CI: 0.87-2.89). Transmission rate parameter estimates were not significantly different between groups 2, 3 and 4. The average estimate of this parameter for the wild-type strain was 4.41 day^{-1} (2.45-7.93). The parameter for the group consisting of V birds was 2.34 day^{-1} (0.91-6.03), for the CV group 8.66 day^{-1} (4.52-16.59) and for groups with NV birds 17.62 day^{-1} (10.94-28.37). After Bonferroni corrections were applied, the transmission rates were not significantly different between V and NV birds (adjusted P = 0.057), V and CV birds (adjusted P = 0.092) or CV and NV birds (adjusted P = 1.000). Significant differences in transmission rates between cages for the wild-type strain were found for group 2a (β = 1.42) compared to 3b (16.11 day^{-1}) and for group 2a compared to 1b (80.40 day^{-1}).
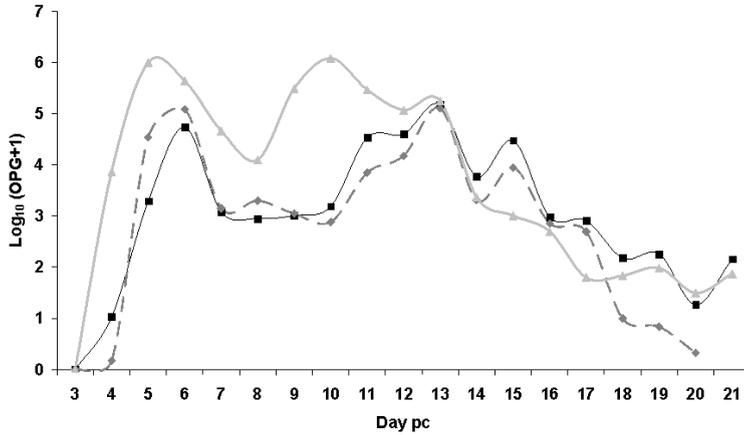
Fig. 3. Mean oocyst excretion in $\log_{10}$ (OPG+1) by vaccinated (—■—), contact-vaccinated (--◆--) and non-vaccinated (—▲—) birds during the challenge period, for inoculated and contact-challenged birds of groups 1a, 1b, 2a, 2b, 3a and 3b combined. Challenge infection with wild-type *E. acervulina* was given to 5 Ino birds of each group of 10 birds at day 0 pc.
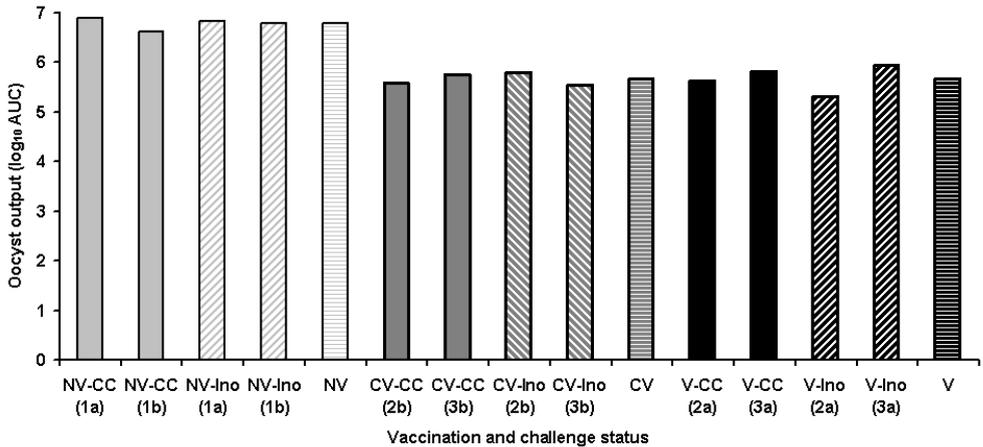
Fig. 4. Total oocyst output ($\log_{10}$ AUC) after challenge infection for non-vaccinated (NV), contact-vaccinated (CV) or directly vaccinated (V) birds. $\log_{10}$ AUC was calculated from output data from days 3-17 pc for inoculated birds (Ino, dashed bars) and days 7-21 pc for contact-challenged birds (CC, solid bars). Horizontally dashed bars show the $\log_{10}$ AUC for Ino and CC birds of all NV, CV or V groups combined. Significant differences in oocyst output were only found for NV groups, compared to CV and V groups ($P < 0.05$). Oocyst output by Ino and CC birds of the same vaccination status (NV, CV and V) were not significantly different ($P > 0.05$).

Table 2
Summary of oocyst output data for the different groups during the vaccination and challenge period.

Category[d]	Status[d]	First peak output[e] Vaccination period Days 3-7 pv (V) / 7-11 pv (CV) Log_{10} AUC (95% CI)	Total output[e] Vaccination period Days 3-23 pv Log_{10} AUC (95% CI)	Total corrected output[e] Vaccination period Days 7-23 pv Log_{10} AUC (95% CI)	Total output[e] Challenge period Days 3-17 pc (Ino) / 7-21 pc (CC) Log_{10} AUC (95% CI)
Group 1 / 1a	NV	-	-	-	6.86 (6.74-6.98)[a]
Group 1 / 1b	NV	-	-	-	6.72 (6.48-6.97)[a]
Group 2 / 2a	V	5.14 (4.98-5.29)[b]	5.82 (5.54-6.10)[ab]	5.66 (5.31-6.00)[ac]	5.47 (5.01-5.93)[b]
Group 3 / 3a	V	4.99 (4.90-5.08)[b]	5.51 (5.28-5.74)[bc]	5.32 (5.04-5.61)[bc]	5.89 (5.67-6.10)[b]
Group 4	V	4.88 (4.70-5.05)[b]	5.40 (5.22-5.58)[bc]	5.15 (4.86-5.44)[c]	-
Group 2 / 2b	CV	5.97 (5.64-6.30)[a]	6.07 (5.80-6.34)[a]	6.07 (5.80-6.34)[a]	5.71 (5.45-5.96)[b]
Group 3 / 3b	CV	5.78 (5.54-6.02)[a]	5.86 (5.65-6.06)[ab]	5.86 (5.65-6.06)[ab]	5.65 (5.42-5.89)[b]
Group 4	CV	5.01 (4.71-5.32)[b]	5.28 (5.03-5.53)[c]	5.28 (5.03-5.53)[bc]	-
Vaccination status	NV	-	-	-	6.80 (6.66-6.93)[a]
Vaccination status	V	5.00 (4.91-5.09)[a]	5.58 (5.43-5.72)[a]	5.38 (5.19-5.57)[a]	5.68 (5.41-5.94)[b]
Vaccination status	CV	5.59 (5.37-5.81)[b]	5.74 (5.56-5.92)[a]	5.74 (5.56-5.92)[b]	5.68 (5.51-5.85)[b]
Challenge status	CC	-	-	-	5.99 (5.74-6.25)[a]
Challenge status	Ino	-	-	-	6.04 (5.79-6.29)[a]

Means and associated 95% Confidence Intervals (CI), calculated based on the normal distribution, are shown.

[a-c] Values within the same column, concerning the same category (group, vaccination or challenge status) with different superscripts differ significantly ($P < 0.05$). General linear models were used to compare oocyst output for the different groups. Linear mixed models, with corrections for random cage effects, were carried out for comparisons of oocyst output for the different vaccination and challenge status.

[d] Groups 1-4 represent group and cage numbers during the vaccination period / Groups 1a, 1b, 2a, 2b, 3a, 3b are group and cage numbers for the challenge period. Birds in group 1 were not vaccinated/contact-exposed during the vaccination period and therefore oocyst output data is only available for the challenge period. Birds of group 4 were not used in the challenge period. Vaccination status refers to non-vaccinated (NV), vaccinated (V) and contact-vaccinated (CV) birds. Challenge status represents birds that were challenged/inoculated with the wild-type strain (Ino) or non-challenged contact-exposed (CC) birds.

[e] Log_{10} AUC, calculated from daily OPG values per bird, was used as a measure for oocyst output for different periods. The periods represent: first excretion peak from days 3-7 pv for V and from days 7-11 pv for CV birds; total oocyst output for the entire vaccination period (days 3-23 pv); total corrected oocyst output for the vaccination period from days 7-23 pv, starting after the first (inoculation-induced) peak of excretion by V birds; total oocyst output for the challenge period from days 3-17 pc for Ino and days 7-21 pc for CC birds.

4. Discussion

The aims of this study were to quantify the transmission rate of an *E. acervulina* vaccine strain in a flock, and furthermore, to compare transmission rates and shedding patterns between vaccinated and contact-vaccinated birds after infection with an *E. acervulina* wild-type strain.

The vaccine strain was transmitted quickly from the orally immunised birds to all non-vaccinated pen mates and it was estimated that, on average, 1.6 naive birds will become vaccinated per day when exposed to a shedding bird. This would imply that if 50% of birds in a flock would ingest the original vaccine dose, more than 95% of the whole flock would become infected with vaccine oocysts within approximately five days after the initial administration of the vaccine. Transmission of the wild-type strain was not significantly reduced, neither among vaccinated nor among contact-vaccinated birds. Furthermore, no significant differences in transmission rates were found between the unvaccinated, vaccinated and contact-vaccinated groups, but the statistical power may have been small. Nevertheless, as the transmission of the wild-type strains occurred so efficiently in all groups, it can be concluded that vaccination did not prevent infection with a wild-type strain.

It was demonstrated, however, that the oocyst output after challenge infection was significantly reduced in both vaccinated and contact-vaccinated birds, compared to unvaccinated birds. This implies that, even if not all birds immediately receive a vaccine dose after mass application, transmission of the vaccine strain might induce an equal level of protection for vaccinated and contact-vaccinated birds against the adverse effects of infection with a wild-type strain. However, as only oocyst output and transmission were determined in this study, further field or experimental work should be done to study the effects on other variables, such as intestinal lesions, performance losses and clinical signs.

It is assumed that in immune hosts, parasites can enter intestinal cells but are prevented from further development (Dalloul and Lillehoj, 2005; Lillehoj et al., 2007) which reduces severity of clinical signs in these birds. Although an individual immune host can still become infected, this does not imply that transmission of infection cannot be reduced, as the probability of birds to become infected in a population is determined by both the infectivity and susceptibility of birds in a flock and the interactions between birds and their environment. In a previous pair-wise transmission experiment (Velkers et al., 2010b) it was demonstrated that both oocyst output and transmission were significantly reduced after challenge infection when both the inoculated and contact birds had been previously infected with a homologous wild-type *E. acervulina* strain. Perhaps, the immune response induced by the homologous wild-type strain can more effectively reduce transmission between birds, compared to the attenuated (precocious) vaccine strain. However, whether this is true and which mechanisms might be involved remains unclear and should be investigated further.

This study showed that the vaccine strain was transmitted rapidly and induced equal levels of protection for vaccinated and contact-vaccinated birds. Therefore, it might seem surprising that outbreaks of clinical coccidiosis and poor performance are frequently reported in

vaccinated broiler flocks. In (vaccinated) broiler flocks, the environmental oocyst load shows a peak, which generally occurs around the fifth week of age, due to circulating wild-type and vaccine strains (Chapman et al., 2002; Williams et al., 2002). Consequently, when the initial exposure to the vaccine is delayed, sufficient protection of a substantial part of the flock may not yet have developed when birds are exposed to heavy challenge doses from the environment, which may result in clinical signs. Furthermore, a delay in exposure might also lead to a shorter remaining time for compensatory growth before slaughter (Henken et al., 1994; Graat et al., 1996), which can further reduce production revenues of the flock. Contact-vaccinated birds become vaccinated later during the flock cycle than the directly vaccinated birds and when initial uptake of vaccine oocysts would be lower, the time before the whole flock is infected increases even further.

It remains unclear which factors determine the efficacy of vaccination in commercial flocks. This study, however, did elucidate that live vaccines have the potential to be efficiently transmitted to initially unvaccinated birds in a flock and that contact-vaccination can induce an equal protection for high oocyst output as direct vaccination after a challenge infection. Further experimental and field studies, aided with mathematical models, are necessary to study the effects of different application methods on initial vaccine intake, transmission, development of protective immunity and production performance for vaccinated flocks.

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Supplementary data

Supplementary Table 1

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique during the vaccination period for group 2.

Group	Bird	Type[a]	Day pv[b] 3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
2	11	V	*-	*+	+*	+*	+*	+*	+*	+*	**	+*	**	+*	**	**	*+	**	**	*-	**	**	*-
2	12	V	*-	*+	+*	+*	+*	+*	+*	+*	**	+*	**	-*	**	**	*+	**	**	*-	**	**	*-
2	21	V	*-	*+	+*	+*	+*	+*	+*	+*	**	-*	**	+*	**	**	*+	**	**	*-	**	**	*-
2	28	V	*-	*+	+*	+*	+*	+*	+*	+*	**	+*	**	+*	**	**	*+	**	**	*+	**	**	*-
2	35	V	*-	*+	+*	+*	+*	+*	+*	+*	**	+*	**	-*	**	**	*+	**	**	*+	**	**	++
2	61	V	*-	*+	+*	+*	+*	+*	+*	+*	**	-*	**	+*	**	**	*+	**	**	*-	**	**	*-
2	76	V	*-	*+	+*	+*	+*	+*	+*	+*	**	+*	**	+*	**	**	*+	**	**	*-	**	**	*-
2	83	V	*-	*+	+*	+*	+*	+*	+*	+*	**	+*	**	-*	**	**	+*	**	**	-+	**	**	*-
2	95	V	*-	*+	+*	+*	+*	+*	+*	+*	**	+*	**	+*	**	**	++	**	**	++	**	**	*-
2	925	V	*+	+*	+*	+*	+*	+*	+*	+*	**	+*	**	+*	**	**	*+	**	**	++	**	**	++
2	926	V	*-	*+	+*	+*	+*	+*	+*	+*	**	+*	**	-*	**	**	*+	**	**	*-	**	**	*-
2	3	CV	**	**	*-	*-	++	+*	+*	+*	+*	+*	+*	**	+*	+*	+*	**	**	+*	**	**	*-
2	18	CV	**	**	*-	-*	*+	*+	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	**	+*	**	**	*-
2	19	CV	**	**	*-	-*	*+	*+	+*	+*	+*	+*	+*	+*	+*	+*	-*	**	**	+*	**	**	*-
2	31	CV	**	**	*-	-*	*-	*+	+*	+*	+*	+*	+*	+*	+*	+*	+*	**	**	+*	**	**	*-
2	34	CV	**	**	*-	--	--	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	**	+*	-*	**	*-
2	51	CV	**	**	*-	-*	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	**	**	+*	**	**	*-
2	63	CV	**	**	*-	-+	*+	*+	+*	+*	+*	+*	+*	+*	+*	+*	+*	**	**	+*	**	**	*-
2	80	CV	**	**	*-	*-	*-	++	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	**	**	-*	**	-+
2	902	CV	**	**	*-	--	*-	*+	+*	+*	+*	+*	+*	+*	+*	-*	+*	**	**	+*	**	**	*-
2	906	CV	**	**	*-	*-	*-	*+	+*	+*	+*	+*	+*	+*	+*	-*	+*	**	**	+*	**	**	*-
2	907	CV	**	**	*-	*-	*-	*+	+*	+*	+*	+*	+*	+*	+*	+*	+*	**	**	-*	**	**	*-

Oocyst output data for each bird: the first symbol represents the results of McMaster oocyst counts (McM), the second symbol the result of sedimentation-flotation slides (SF): +: oocysts were detected, *: not done, -: no oocysts were detected. Dark grey marked sections represent McM results above zero. Light grey marked sections represent samples that had positive SF results but had missing or negative McM values.

[a] Vaccinated birds (V) received 600 sporulated *E. acervulina* oocysts of the Paracox vaccine at day 0 pv, contact-vaccinated (CV) birds were sham-vaccinated with tap water and were contact-exposed to vaccinated birds from day 1 pv onwards.

[b] Days after vaccination at day 2 of age (day 0 pv).

Supplementary Table 2
Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique during the vaccination period for group 3.

Group	Bird	Type[a]	Day pv[b] 3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
3	6	V		* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	- *	* *	* *	* +	* *	* *	- +	* *	* *	+ +
3	7	V		* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *
3	25	V		* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	* *	+ +	* *	* *	+ +	* *	* *	+ +
3	43	V		* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	* *	* -	* *	* *	* -	* *	* *	* -
3	52	V		* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* *	- *	* *	* *	* +	* *	* *	* -	* *	* *	+ +
3	64	V		* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* *	+ *	* *	* *	- +	* *	* *	* +	* *	* *	+ +
3	70	V		* -	* +	+ *	- *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	* *	* *	* *	* *	* -	* *	* *	* +
3	74	V		* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	- *	* *	* *	- +	* *	* *	+ +	* *	* *	* -
3	75	V		* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	- *	* *	* *	* +	* *	* *	+ +	* *	* *	* -
3	912	V		* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* *	+ *	* *	* *	* -	* *	* *	* -	* *	* *	* -
3	928	V		* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	* *	+ +	* *	* *	+ +	* *	* *	- +
3	0	CV	* *	* *	* -	- -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	* *	* *	- *	* *	* *	+ +
3	8	CV	* *	* *	* -	+ +	* +	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	* *	+ *	* *	* *	+ +
3	30	CV	* *	* *	* -	* +	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	+ *	* *	+ *	* *	* *	+ +
3	32	CV	* *	* *	* -	* -	* +	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	* *	* *	- *	* *	* *	* *
3	45	CV	* *	* *	* -	* -	- -	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	- *	* *	* *	* -
3	47	CV	* *	* *	* -	+ +	+ +	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	- *	* *	* *	+ +
3	53	CV	* *	* *	* -	* -	* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	- *	* *	* *	* -
3	56	CV	* *	* *	* -	* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	* *	* *	- *	* *	* *	* -
3	62	CV	* *	* *	* -	- -	* +	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	+ *	* *	* *	* -
3	81	CV	* *	* *	* -	* -	* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* -
3	88	CV	* *	* *	* -	- -	* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	- *	* *	* *	+ +

Oocyst output data for each bird: the first symbol represents the results of McMaster oocyst counts (McM), the second symbol the result of sedimentation-flotation slides (SF): +: oocysts were detected, *: not done, -: no oocysts were detected. Dark grey marked sections represent McM results above zero. Light grey marked sections represent samples that had positive SF results but had missing or negative McM values.
 [a] Vaccinated birds (V) received 600 sporulated *E. acervulina* oocysts of the Paracox vaccine at day 0 pv, contact-vaccinated (CV) birds were sham-vaccinated with tap water and were contact-exposed to vaccinated birds from day 1 pv onwards.
 [b] Days after vaccination at day 2 of age (day 0 pv).

Supplementary Table 3

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique during the vaccination period for group 4.

Group	Bird	Type[a]	Day pv[b] 3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
4	5	V	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* *	- *	* *	* *	* +	* *	* *	* +	* *	* *	* -
4	23	V	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* *	- *	* *	* *	* +	* *	* *	* +	* *	* *	* -
4	40	V	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* *	- *	* *	* *	* -	* *	* *	+ +	* *	* *	- +
4	60	V	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* *	- *	* *	* *	* +	* *	* *	+ +	* *	* *	* -
4	84	V	* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	* *	* +	* *	* *	- +	* *	* *	* -
4	85	V	* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* *	+ *	* *	* *	* +	* *	* *	* -	* *	* *	- +
4	87	V	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	- *	* *	* *	* +	* *	* *	- +	* *	* *	- +
4	96	V	* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	* *	* -	* *	* *	* -	* *	* *	- +
4	910	V	* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	* *	* +	* *	* *	- +	* *	* *	* -
4	921	V	* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* *	- *	* *	* *	+ +	* *	* *	* -	* *	* *	* +
4	922	V	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	* *	* +	* *	* *	* +	* *	* *	* -
4	10	CV	* *	* *	* -	* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	- *	* *	* *	* -
4	15	CV	* *	* *	* -	* -	* +	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	- +
4	38	CV	* *	* *	* -	* -	- -	* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* -
4	48	CV	* *	* *	* -	* -	* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	* *	* *	- *	* *	* *	* -
4	54	CV	* *	* *	* -	* -	* +	* +	+ *	+ *	+ *	+ *	+ *	- *	+ *	- *	* *	* *	- *	* *	* *	* *	- +
4	59	CV	* *	* *	* -	* -	* -	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- +
4	73	CV	* *	* *	* -	* -	- +	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* *	* *	* -
4	97	CV	* *	* *	* -	* -	* +	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	* *	* *	+ *	* *	* *	* -
4	98	CV	* *	* *	* -	* -	* -	* +	+ *	+ *	+ *	- *	+ *	- *	+ *	+ *	- *	* *	* *	- *	* *	* *	* -
4	903	CV	* *	* *	* -	* -	* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	+ *	* *	* *	+ +
4	917	CV	* *	* *	* -	* -	* -	* +	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	* *	* *	+ +

Oocyst output data for each bird: the first symbol represents the results of McMaster oocyst counts (McM), the second symbol the result of sedimentation-flotation slides (SF): +: oocysts were detected, *: not done, -: no oocysts were detected. Dark grey marked sections represent McM results above zero. Light grey marked sections represent samples that had positive SF results but had missing or negative McM values.

[a] Vaccinated birds (V) received 600 sporulated *E. acervulina* oocysts of the Paracox vaccine at day 0 pv, contact-vaccinated (CV) birds were sham-vaccinated with tap water and were contact-exposed to vaccinated birds from day 1 pv onwards.

[b] Days after vaccination at day 2 of age (day 0 pv).

Supplementary Table 4

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique during the challenge period for the non-vaccinated, challenged birds of groups 1a and 1b.

Group	Bird	Type[a]	Day pc[b]	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1a	37	NV1-Ino		* -	* -	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	- *	* *	+ *	* *	- -	* *
1a	39	NV1-Ino		* -	* *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	* *	* *	+ *	+ *
1a	99	NV1-Ino		* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *
1a	911	NV1-Ino		* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *
1a	932	NV1-Ino		* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- *	* *	* *	+ *
1a	41	NV1-CC		* -	* *	* *	* *	+ +	+ *	+ *	+ *	* *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	+ *	* *
1a	44	NV1-CC		* -	* *	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	- +	* *
1a	58	NV1-CC		* -	* *	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	+ *	* *
1a	930	NV1-CC		* *	* -	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	+ *	- +
1a	933	NV1-CC		* -	* *	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	- *	* *
1b	1	NV1-Ino		* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- *	* *	+ +	* *
1b	4	NV1-Ino		* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- *	* *	* -	* *
1b	17	NV1-Ino		* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *
1b	24	NV1-Ino		* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- *	* *
1b	33	NV1-Ino		* -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	+ *
1b	69	NV1-CC		* -	* *	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	+ *	- *	* *	- *	* -	* *
1b	71	NV1-CC		* -	* *	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	* *	- *	+ *	+ *
1b	909	NV1-CC		* -	* *	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	* *	+ *	+ *	* *
1b	914	NV1-CC		* -	* *	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	* *	* *	* *	* *	* *	* *
1b	916	NV1-CC		* -	* *	* *	* *	+ +	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *

Oocyst output data for each bird: the first symbol represents the results of McMaster oocyst counts (McM), the second symbol the result of sedimentation-flotation slides (SF): +: oocysts were detected, *: not done, -: no oocysts were detected. Dark grey marked sections represent McM results above zero. Light grey marked sections represent samples that had positive SF results but had missing or negative McM values.

[a] Inoculated birds (Ino) received a challenge infection with 5000 sporulated *E. acervulina* oocysts of a wild-type (Weybridge) strain at day 0 pc, contact-challenged (CC) birds were not inoculated and were contact-exposed to challenged birds from day 1 pc onwards.

[b] Days after challenge at day 28 of age (day 0 pc).

Supplementary Table 5

Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique during the challenge period for the vaccinated (2a) and contact-vaccinated (2b) birds of group 2.

Group	Bird	Type[a]	Day pc[b]	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
2a	11	V2-Ino		* -	* -	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	- *	* *	- *	* *	- -	* *
2a	61	V2-Ino		* -	* -	* -	+ +	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- +	* *
2a	76	V2-Ino		* -	+ +	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- +	* *
2a	83	V2-Ino		* -	* -	* -	+ +	+ *	+ +	+ *	+ *	* *	+ *	* *	+ *	* *	* *	* *	+ *	* *	- *	* -
2a	925	V2-Ino		* -	* -	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- -	* *
2a	12	V2-CC		* -	* *	* *	* *	* -	* -	- -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	+ *	* *
2a	21	V2-CC		* -	* *	* *	* *	* -	- +	- -	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	+ *	- -
2a	28	V2-CC		* *	* -	* *	* *	* -	- -	- -	* -	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	+ *	+ *
2a	35	V2-CC		* -	* *	* *	* *	+ +	- +	- -	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	- -	* *
2a	95	V2-CC		* -	* *	* *	* *	* -	- +	+ +	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	- +	* *
2b	31	CV2-Ino		* -	* -	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- -	* *
2b	51	CV2-Ino		* -	+ +	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- -	* *
2b	902	CV2-Ino		* -	* -	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- *	* *	+ +	* *
2b	906	CV2-Ino		* -	- +	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	- *	* *	+ *	* *	- -	* *
2b	907	CV2-Ino		* -	- +	+ +	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	+ *	* *	+ *	* *	- *	* *	- +	* *
2b	3	CV2-CC		* -	* *	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	- -	* *
2b	18	CV2-CC		* -	* *	* *	* *	* -	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	+ *	* *
2b	19	CV2-CC		* -	* *	* *	* *	+ +	- *	- -	- -	* +	+ *	+ *	+ *	+ *	+ *	- *	* *	- *	- -	* *
2b	34	CV2-CC		- -	* *	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	- *	* -	* *
2b	80	CV2-CC		* -	* *	* *	* *	+ +	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *	* *

Oocyst output data for each bird: the first symbol represents the results of McMaster oocyst counts (McM), the second symbol the result of sedimentation-flotation slides (SF): +: oocysts were detected, *: not done, -: no oocysts were detected. Dark grey marked sections represent McM results above zero. Light grey marked sections represent samples that had positive SF results but had missing or negative McM values.

[a] Inoculated birds (Ino) received a challenge infection with 5000 sporulated *E. acervulina* oocysts of a wild-type (Weybridge) strain at day 0 pc, contact-challenged (CC) birds were not inoculated and were contact-exposed to challenged birds from day 1 pc onwards.

[b] Days after challenge at day 28 of age (day 0 pc).

Supplementary Table 6
Oocyst output results of the McMaster counting chamber and sedimentation-flotation technique during the challenge period for the vaccinated (3a) and contact-vaccinated (3b) birds of group 3.

Group	Bird	Type[a]	Day pc[b]	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
3a	6	V3-Ino		* -	+ +	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *
3a	25	V3-Ino		* -	+ +	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	+ *
3a	64	V3-Ino		* -	* -	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *
3a	70	V3-Ino		* -	- +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- *	* *
3a	912	V3-Ino		* -	- +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- -	* *
3a	43	V3-CC		* -	* *	* *	* *	+ +	- +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	- *	* *
3a	52	V3-CC		* -	* *	* *	* *	+ +	* +	+ *	+ *	+ *	* *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *
3a	74	V3-CC		* -	* *	* *	* *	+ +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *
3a	75	V3-CC		* *	* -	* *	* *	+ +	+ *	- -	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	- *	* *	+ *	* *
3a	928	V3-CC		* -	* *	* *	* *	+ +	* +	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	+ *
3b	0	CV3-Ino		* -	* -	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	- *	* *	- -	* *
3b	8	CV3-Ino		* *	* -	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	* *	+ *	* *	+ *	* *	- *	* *	- +	* *
3b	47	CV3-Ino		* -	* -	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	- *	* *	+ *	* *	+ *	* *	- -	* *
3b	62	CV3-Ino		* -	- +	+ +	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *	+ *	* *
3b	81	CV3-Ino		* -	* -	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	* *	+ *	* *	- *	* *	- +	* *
3b	30	CV3-CC		* -	* *	* *	* *	- +	- -	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	* *	- +	* *
3b	45	CV3-CC		* -	* *	* *	* *	+ +	- +	+ *	+ *	* +	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	- -	* *
3b	53	CV3-CC		* -	* *	* *	* *	+ +	* *	+ *	+ *	+ *	* *	+ *	+ *	+ *	+ *	+ *	* *	+ *	- -	* *
3b	56	CV3-CC		* -	* *	* *	* *	* -	- +	- -	+ *	+ *	* *	+ *	+ *	+ *	+ *	+ *	* *	- *	- +	* *
3b	88	CV3-CC		* -	* *	* *	* *	- +	- -	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	+ *	* *	+ *	- *	* *

Oocyst output data for each bird: the first symbol represents the results of McMaster oocyst counts (McM), the second symbol the result of sedimentation-flotation slides (SF): +: oocysts were detected, *: not done, -: no oocysts were detected. Dark grey marked sections represent McM results above zero. Light grey marked sections represent samples that had positive SF results but had missing or negative McM values.

[a] Inoculated birds (Ino) received a challenge infection with 5000 sporulated *E. acervulina* oocysts of a wild-type (Weybridge) strain at day 0 pc, contact-challenged (CC) birds were not inoculated and were contact-exposed to challenged birds from day 1 pc onwards.

[b] Days after challenge at day 28 of age (day 0 pc).

Chapter 7

General Discussion

7.1. Introduction

The aim of this thesis was to provide more insights into the transmission dynamics of an infection with *Eimeria acervulina* in broiler flocks by unravelling underlying mechanisms that can affect the course of infection. This may facilitate the development and improvement of control strategies for coccidiosis.

Pair-wise transmission experiments showed that oocyst output increased with higher ingested doses of *E. acervulina,* but it also showed that the transmission rate was independent of the ingested dose (*chapter 3*). Furthermore, contact-infected birds excreted more oocysts than inoculated birds. Results from the group experiments were consistent with experiments from pairs; the excreted dose significantly increased during successive generations of infection, but the transmission rate remained constant (*chapter 5*). When birds had experienced a primary infection before the start of a pair-wise transmission experiment, oocyst output was significantly reduced after a secondary infection compared to non-primed birds. Transmission was significantly reduced when both the inoculated and contact-exposed bird had been primed (*chapter 4*).

Vaccination is one of the alternative intervention measures, but is not often used in broilers. Optimisation of vaccination strategies might contribute to more frequent use of vaccines in this poultry sector. In *chapter 6* it was shown that a live vaccine strain of *E. acervulina* was efficiently transmitted to unvaccinated birds. Broilers inoculated with vaccine and those that became contact-vaccinated (due to exposure to vaccine oocysts excreted by vaccinated birds) both excreted significantly lower numbers of oocysts after an infection with a wild-type strain of *E. acervulina*, but transmission of the wild-type strain was not significantly reduced compared to birds that had not been (contact)-vaccinated.

In this chapter, the potential feasibility and efficacy of different targets for coccidiosis control will be discussed, in light of the knowledge of transmission dynamics acquired with this thesis. Furthermore, this chapter will conclude with recommendations for further research to study transmission dynamics and efficacy of control measures and with a summary of the main conclusions of this thesis.

7.2. Potential targets for the control of coccidiosis

Potential strategies for the control of *Eimeria* infections in the poultry industry can have distinctive primary targets:

1) Reduction of exposure to infectious oocysts or developing stages in the intestine;
2) Improvement of the immune response in the hosts;
3) Reduction of transmission within a flock;
4) Reduction of transmission between flocks.

The most often applied strategy for broiler flocks currently involves using anticoccidials in the feed throughout the production period. In addition, most farms apply strict biosecurity measures to reduce the numbers of oocysts at the start of a new flock. Prophylactic use of anticoccidials impedes the development of the parasite in the intestine, and consequently, reduces clinical signs and oocyst exposure for other birds in the flock. Although primarily aimed at reducing exposure to oocysts or other parasite stages, anticoccidials in the feed also allow for some build up of immunity (Chapman, 1999a; Chapman, 1999b).

Vaccination with live *Eimeria* strains, which is currently one of the most promising alternatives for anticoccidials, primarily aims at inducing a quick build up of immunity to protect against infection with circulating wild-type strains. These strategies are mainly aimed at reducing clinical symptoms of coccidiosis and production losses. However, as most of these approaches affect the infectivity and susceptibility of birds, transmission within a flock, and perhaps between subsequent flocks or farms, might also be affected and perhaps may be applicable as primary targets. For sustainable control of coccidiosis in the future, further research and evaluation of the efficacy and cost-effectiveness of interventions, based on these potential targets, is warranted.

7.2.1. Exposure to oocysts in infected flocks

Oocyst shedding patterns during a flock infection
It is assumed that the infection chain starts with a few birds that ingest very small doses of oocysts (Long and Rowell, 1975; Reyna et al., 1983; Williams, 1995a). After infection of the first bird(s), the oocyst load in the environment increases during further spread of the infection in the flock. During field studies, in which oocyst load was determined throughout broiler flock production cycles, litter oocyst counts were generally low during the first two weeks and reached a peak at four or five weeks of age, after which oocyst load declined to low levels towards the end of the grow-out at six to eight weeks (Reyna et al., 1983; Chapman et al., 2002; Williams, 2002). This would imply that chickens that become infected later during the production cycle are more likely to ingest higher oocyst doses than the first birds in the infection chain, which can result in a higher oocyst output and may lead to more severe signs in these chickens.

An increase in oocyst output, indicating ingestion of a higher oocyst dose from the environment (Williams, 2001), was also observed for successive generations of infection during the transmission experiments. Contact-infected chickens excreted more oocysts than their inoculated pen mates (*chapter 3*) and "second generation" contact-infected birds that became infected later shed significantly more oocysts than "first generation" contact birds (*chapter 5*). These results suggest that the timing of infections for individual birds in the flock can have a substantial impact on symptoms in individual hosts, the dynamics of oocyst build up in the environment, and consequently on oocyst exposure for other birds in the population and flock performance, as explained further below.

Oocyst load and flock performance

Infection of the flock will not always result in a substantial effect on performance and clinical signs. Previous studies have shown that it might not be beneficial to start a broiler grow-out without the presence of low numbers of oocysts in the poultry house. For instance, the "new house syndrome" might occur when birds are reared in a "coccidia-free" house: because of the absence of immunity inducing (repeated) infections with low oocyst doses, more severe signs can occur when birds are infected later in life (Shirley, 1992; McDougald, 2008). Graat et al. (1996) and Henken et al. (1994) indicated that when broiler flocks were exposed to an intermediate initial contamination level of the litter, production performance was better compared to a high or low initial oocyst load. They hypothesised that when the oocyst contamination level at the start of a flock cycle is low, the peak in oocyst load and the associated depression in production performance occur later during the production cycle. At a high initial contamination level, the oocyst load remains high throughout the flock cycle and performance depression persists for a longer period of time. It is assumed that in both cases, the time for compensatory growth before slaughter and economic revenues would be reduced compared to flocks starting with an intermediate contamination level.

Similar infection dynamics were described for other mathematical models on *Eimeria*, which indicated that both low and high contamination levels can result in a higher maximum infection load within a cohort (Klinkenberg and Heesterbeek, 2007) or occurrence of high dose excretions by individuals in a flock (Severins et al., 2007), which were assumed to be associated with the severity of an outbreak and production losses. An important observation in this context is that there is a considerable difference in the adverse effects of oocyst exposure at the level of the population compared to the individual host. On population level, the oocyst load in the environment and the clinical outcome are not linearly related and production losses may be higher when the infection process starts with very low or high initial contamination levels. On the level of the host, however, oocyst input and output are highly related with each other and to the occurrence of clinical symptoms. A higher oocyst dose generally results in a higher oocyst excretion and more severe clinical symptoms, provided that these doses remain below the crowding threshold, above which oocyst output decreases with a higher ingested dose (Williams, 2001). Therefore, the severity of an *Eimeria* infection, as manifested by intestinal lesions, mortality, and depression of weight gain, feed intake and feed efficiency, generally increases with the excreted oocyst dose.

A significant effect of oocyst exposure on body weight or mortality was not found during the studies in this thesis and other production-related variables were not measured. For commercial flocks, small effects on weight gain, feed efficiency and mortality can have considerable effects on revenues. Detection of these effects, however, would require larger experimental groups than used for the transmission experiments. Other factors that can influence the host response to infection include the species/strain of *Eimeria*, concurrent infections, nutritional status, age of the birds and immune status (Hein, 1968; Long, 1984; Lillehoj, 1988; Conway et al., 1993; Zhu et al., 2000; McDougald, 2008). This illustrates that

infection dynamics of *Eimeria* infections in a flock can be very complex and that effects of interventions on infection dynamics and the clinical outcome in a flock may be unpredictable and sometimes even counterintuitive.

Oocyst load and transmission
Transmission of an infectious agent depends on the infectivity of infected birds, susceptibility of not yet infected birds, and the interaction between these two types of birds and their contaminated environment. Although the relations between oocyst level and performance of the birds have been described for field experiments and have been studied using mathematical models (Henken et al., 1994; Graat et al., 1996; Klinkenberg and Heesterbeek, 2005; Klinkenberg and Heesterbeek, 2007; Severins et al., 2007), the underlying processes were not elucidated then. In this thesis, transmission experiments were used to obtain insights into the relative contribution of different factors to the transmission dynamics of *Eimeria.*

First, the relation between oocyst exposure levels and the transmission rate of *E. acervulina* was studied. In *chapter 3* it was shown that the transmission rate was not significantly affected by the oocyst inoculation dose. This finding was unexpected, as the oocyst output by the inoculated bird increased substantially with a higher inoculation dose. In agreement with these trials, it was shown in group experiments (*chapter 5*) that the excreted oocyst dose increased for successive generations, but the transmission rate remained constant. These experiments therefore provided a novel outlook on transmission dynamics, i.e. that not the actual number of oocysts excreted in the environment, but most likely the probability of coming into contact with infectious faecal material, determines the transmission rate.

A theory that may explain a potential underlying process of transmission dynamics was introduced in *chapter 5*. The comparisons of transmission parameters from the pair-wise and group experiments and simulation models suggested that the infectivity of infectious birds is spread out over several days, after which a level of maximum infectivity is reached. The infectivity builds up as the surface of the litter is increasingly covered with infectious faecal droppings, excreted by the infectious chicken. After a few days, the oocysts in these droppings may become unavailable for ingestion, either due to subsiding to lower levels of the litter or disintegration due to ammonia and bacteria. Consequently, infectivity increases until equilibrium is reached in the litter between the increase and decrease of infectivity. For the transmission experiments it was assumed that the infectivity increased over four days, as this fitted the experimental data. This suggests that, especially at the start of a flock infection when only a few chickens are excreting contaminated faeces, the transmission rate might gradually increase over a few days to a plateau level, at which it remains constant for as long as the number of infectious individuals remains constant. For transmission experiments with *Eimeria,* and perhaps also for other pathogens, the mathematical models used for the estimation of the transmission rate should correct for the effects of this build up phase.

The findings of this thesis indicate that not the oocyst load in the environment, but other factors may affect transmission of the parasite. It has been shown that very low oocyst doses (*chapters 2, 3 and 5*), or even a single ingested oocyst, can establish an infection (Norton and Joyner, 1986). Therefore, it is not unlikely that transmission is mostly determined by the probability of coming into contact with infectious faecal material, irrespective of the total number of oocysts present in this faecal material. Therefore, transmission can be associated with the degree and dispersal of contamination with faecal material in the environment. Some strictly hypothetical examples of targets to affect transmission of infection include: affecting the distribution of faeces and exposure of birds to faecal material by changing density/flock size or by changing the location of heaters, feed or water equipment to affect movement patterns of birds; reducing the total volume of droppings by adjusting the feed; influencing dispersal and survival of oocysts in different layers of the litter by changing litter type, applying additives to the feed or litter or by adjusting the local litter conditions/climate; changing litter pecking behaviour by offering other pecking substrates, etcetera. Another example is housing broilers on wired floors instead of litter. Affecting movement patterns of chickens and cage housing will be discussed further in the following paragraph. The exact effects of these factors on transmission dynamics are unknown and most factors are currently impractical as control targets. However, it may also be important to consider the influence of these factors in future research on optimising control strategies.

Effects of farm management on oocyst load
Farm management should be aimed towards optimising the basic requirements for health and welfare of chickens, e.g. feed, water and environmental conditions, to support the hosts' combat against disease (Allen and Fetterer, 2002; Peek, 2010). Management procedures that might have a direct influence on oocyst levels include those related to climate and litter conditions, housing and behaviour of the birds.

Climatic and litter conditions can influence the oocyst load in the environment, but the range in temperature, humidity and ventilation beneficial for the chickens are also favourable for survival and sporulation of oocysts (Edgar, 1955; Graat et al., 1994; Waldenstedt et al., 2001). Consequently, changing these conditions within the small range acceptable for health and performance of the chickens most likely has only minimal effects on oocysts load.

Rearing chickens on cages significantly reduces contact with faeces and consequently reduces exposure to oocysts. Cage-reared flocks even can remain coccidia-free, when strict biosecurity measures are applied. Welfare considerations and legislation will, however, most likely reduce further use of cage systems (Long, 1984).

Bird density might affect movements of birds, and consequently, dispersal and contact with contaminated faecal droppings in the environment. Studies describing movement of broilers through their confinements are limited and often show contradictory results. Newberry and Hall (1988) reported no effects of bird density on use of space, but other studies suggested that increased bird density reduced the mean distances travelled by chicks

(Lewis and Hurnik, 1990; Newberry and Hall, 1990). Nevertheless, movements were quite random (Preston and Murphy, 1989; Arnould and Faure, 2002), not socially restricted to any particular areas (Preston and Murphy, 1989; Lewis and Hurnik, 1990) and the birds moved over larger areas than necessary to obtain feed, water and warmth (Newberry and Hall, 1988; Newberry and Hall, 1990). This implies that oocysts are likely to be fairly evenly spread and encountered throughout a broiler house (Williams, 2002). Changing the placement of heaters, feed and water equipment might affect broiler movements and may be applicable for commercial farms to manipulate the dispersal of and exposure to infected faeces. Reducing the flock size and/or bird density might affect infection dynamics, but also has a considerable impact on the economic revenues of the flock and therefore might not be a short term feasible target for intervention measures. Furthermore, the association between bird density and infection dynamics at different times during a flock cycle should be studied further, as it might even be beneficial to increase bird density during certain periods of the grow-out to facilitate immunity inducing re-infections or after vaccination, to facilitate transmission of the vaccine strain.

Oocyst exposure and control strategies
Although ingested oocyst dose and transmission were not associated and although the initial oocyst load in the environment might have a parabolic association with the severity of signs, the probability for birds to come into contact with high oocyst doses, throughout the flock cycle, should be avoided. When oocyst loads during a flock infection are very high, the initial contamination level for a subsequent flock might exceed the optimum (intermediate) contamination level and might reduce flock performance. Furthermore, when a substantial number of chickens ingest a high oocyst dose, these birds will develop clinical signs, which might adversely affect the overall performance of the flock. To reduce the occurrence of high doses, current intervention procedures include prophylactic use of anticoccidial drugs or vaccination to reduce multiplication of the parasite and oocyst output by infected birds. Furthermore, these measures and biosecurity procedures additionally aim at reducing high initial contamination levels for other flocks. However, despite these attempts outbreaks still occur in poultry flocks, due to the waning efficacy of anticoccidial drugs and variable efficacy of vaccination protocols.

This thesis has revealed some potential underlying mechanisms that determine infection dynamics and that warrant further research and might eventually improve the efficacy of current strategies or identify novel approaches. In addition to measures that aim at reducing exposure to oocysts, control efforts directed at reducing transmission within and between flocks, as will be discussed further below, might be efficacious, and should be considered. Because transmission of infection is most likely not determined by the total oocyst load, but possibly by the probability for birds to come into contact with infectious faeces, another (novel) approach might be to attempt to influence this process. Some examples of factors that can possibly influence the exposure to infectious material have been suggested in the

preceding paragraphs. However, efficacy for the control of coccidiosis, practicality and cost-effectiveness of these measures and (side)-effects for health, production performance and welfare of the flock should be carefully studied and evaluated.

7.2.2. Development of the parasite in the host

The most frequently applied control strategy that is primarily aimed towards reducing the development of the parasite in the host is the use of anticoccidial drugs. The drugs are mostly applied prophylactically via medicated feed or therapeutically during outbreaks. Coccidiostatic drugs arrest the development of certain parasite stages and coccidiocidal drugs kill or irreversibly damage most of the parasite stages. Preventive medication in the feed has been a convenient, labour-saving cost-effective method, but due to its abundant use for many decades resistance has developed to all the anticoccidial drugs introduced so far. To minimise the effects of resistance, poultry producers use various anticoccidials with successive flocks ("rotation") or use two or more different anticoccidial drugs during a flock grow-out (Allen and Fetterer, 2002). On broiler farms with regular outbreaks due to resistant *Eimeria* strains, live coccidiosis vaccines are included in these rotation programmes in an attempt to increase sensitivity to anticoccidial drugs for subsequent flocks (Mathis and Broussard, 2006; Peek and Landman, 2006). This strategy cannot be applied anymore in the European Union in the near future, because a total ban of prophylactic use of anticoccidials is likely to be implemented by 2012 (Shirley et al., 2005).

Anticoccidial treatment impedes the multiplication of the parasite and consequently reduces oocyst output, intestinal lesions and adverse effects of an infection on weight gain and feed efficiency (McDougald, 2008). As none of the anticoccidials succeed in completely preventing parasite multiplication, some protective immunity can develop in treated birds (Chapman, 1999a; Chapman, 1999b). In medicated flocks oocysts are present in the intestines of birds and are also found in the litter. The peak output in medicated flocks is lower compared to unmedicated flocks, but is generally higher compared to successfully vaccinated flocks (Williams et al., 1999).

7.2.3. Immunity and Eimeria infections

Response of the immune system to an Eimeria infection

Coccidia are highly immunogenic and a primary infection can stimulate solid immunity to homologous infection. The immune response can reduce clinical signs and oocyst output in individual birds after infection and reduces susceptibility of birds for infection. Consequently, the immune response can have a significant impact on infection dynamics in a flock. Therefore, more knowledge of interactions between the parasite and the immune response of the host and effects on transmission in a flock might be important for coccidiosis control.

Immune responses to *Eimeria* involve both innate immunity and acquired immunity. The latter is mediated by antibodies, lymphocytes and cytokines and includes both cellular and humoral immune mechanisms. Early endogenous stages of the *Eimeria* life cycle are considered to be most important for the induction of protective immune responses. In immune hosts parasites enter the gut early after infection but are prevented from further development, indicating that acquired immunity to coccidiosis may involve mechanisms that inhibit the natural progression of parasite development (Dalloul and Lillehoj, 2005; Lillehoj et al., 2007).

Protective immunity is influenced by host genetic background, parasite species/strain and infection history of the host: protection against re-infection tends to improve with an increasing initial oocyst dose (Hein, 1976; Rose, 1987; Lillehoj, 1988; Blake et al., 2005) and with repeated infections (Hein, 1975; Joyner and Norton, 1976; Galmes et al., 1991; Stiff and Bafundo, 1993; Chapman and Cherry, 1997; Blake et al., 2005; Velkers et al., 2010). Host immunity is *Eimeria* species-specific and little cross-protection is observed against heterologous species and, in some cases, cross-protection is even absent for different strains of the same species (Lillehoj and Lillehoj, 2000).

The immune response and current control strategies for coccidiosis
The most important control strategies that target the immune response include immunomodulation and vaccination. Immunomodulators for *Eimeria* are mostly aimed towards influencing mucosal physical integrity and the immune system and include vitamins and microminerals, natural products (e.g. betaine), probiotics, mushroom extracts, synthetic oligonucleotides, cytokines and antibodies.

Vaccines have been available for over 50 years and mostly consist of live vaccines with unattenuated or attenuated strains of *Eimeria*. Recombinant (DNA or protein) vaccines have been developed as more cost-effective and safer alternatives for live vaccines and recently much work has been done on developing adjuvants to further enhance immunogenicity of vaccines (Allen and Fetterer, 2002; Dalloul and Lillehoj, 2005; Dalloul and Lillehoj, 2006). In addition to vaccinating chickens on the farm, specific applications include *in ovo* delivery of sporulated oocysts (Weber et al., 2004) or of a DNA vaccine (Ding et al., 2005) and immunisation of breeders for antibody transfer to the offspring (Wallach, 2010).

The most often used vaccines up to now are live attenuated or non-attenuated vaccines, which are frequently used in layer and breeder flocks. The use in broilers remains limited, however, due to relatively high costs of the vaccine in relation to the small profit margins, doubts about the timely onset of protective immunity in birds with such a short life span and concerns about adverse effects on growth (Williams, 2002).

Effects of immunity on host and population
For the individual bird, protective immunity results in reduced oocyst output and improved production performance (Holdsworth et al., 2004).

On a population level, immunity of individual birds can affect the transmission of the parasite between birds. A simulation model, where the effects of infection history, immunity and movements of individual birds on the spread of *E. acervulina* were studied, showed that (stochastic) differences in dose and frequency of oocyst ingestions from the environment during the course of infection induced heterogeneity in exposure history and immune status in the population (Severins et al., 2007). Increasing the homogeneity in immune status of individual birds in a flock reduced the severity of the outbreak and clinical signs, according to this model.

Pair-wise transmission experiments were carried out to study the influence of a previous infection history on immune parameters, oocyst output and transmission (*chapter 4*). Different combinations of primed inoculated or primed contact-exposed chickens were used. When only the inoculated bird was primed, transmission to the contact-exposed bird and subsequent oocyst excretion after contact-infection were hardly affected. When the inoculated bird was non-primed and the contact-exposed bird was primed, oocyst output was significantly lower compared to non-primed contacts, but nevertheless all contact birds became infected. When both the inoculated and contact bird had been primed, however, transmission only occurred in one of the six pairs and oocyst output was hardly detectable. The combination of the reduced infectivity and reduced susceptibility resulted in a significant reduction of the transmission. It cannot be determined whether the five contact birds did not become infected at all or that a low grade infection was present but might have been undetected. Nevertheless, if infection did occur, the "primed" immune system most likely quickly arrested the development of any invading parasites, and therefore, the transmission between birds was significantly reduced.

Efficacy of vaccination: a race between protective immunity and challenge infections
Because of the short life span of broilers the available time for most individuals in the flock to develop protective immunity is limited (Williams, 2002). The oocyst load in the litter usually shows a peak from four or five weeks of age onwards in standard, prophylactically medicated, broiler flocks. In vaccinated flocks, the oocyst load also increases during the flock cycle and usually two peaks are observed, which are usually lower than the single peak in medicated flocks. Several authors suggested that the first peak is due to replication of the vaccine strain and the second peak, around the fifth week of age, due to circulating wild-type oocysts (Chapman et al., 2002; Williams, 2002). Furthermore, it is also suggested that an effective vaccine should provide protective immunity for a large part of the flock before the chickens are exposed to heavy challenge doses during these peaks in oocyst load. This would imply that a delay in exposure to the vaccine by individual birds in a flock would hamper the immune system in this race against the increasing environmental load, which may result in disease in insufficiently protected birds that ingest a high oocyst dose.

Based on the transmission rate of the live vaccine studied in this thesis (*chapter 6*), it is highly unlikely that birds that have not become infected with vaccine oocysts are present

when the oocyst load reaches a high level. However, as it is assumed that it takes at least 14 days before birds are protected against a challenge infection after exposure to vaccine oocysts (Williams, 1992; Williams, 1995b; Williams, 2002; Crouch et al., 2003), it is not unlikely that the immune system can loose this race against the increasing oocyst load, despite quick contact-vaccination of all birds in a flock. Another explanation might be that a delay in exposure may lead to a shorter remaining time for compensatory growth after the infection with (vaccine) oocysts before slaughter, which can further reduce production revenues of the flock (Henken et al., 1994; Graat et al., 1996).

Consequently, aiming towards inducing protection of a large part of a flock as soon as possible is important, especially for broiler flocks. Therefore, it has often been suggested that a crucial aspect for vaccine efficacy of live vaccines is the uniformity of uptake of the intended oocysts dose by a large proportion of birds in the flock. It is assumed that this is often not fully achieved due to the mass application of live vaccines in broiler flocks (Chapman et al., 2002; Williams, 2002; Shirley et al., 2005).

Another factor that is assumed to be crucial for efficacy is the immune response stimulating effect of multiple re-infections with oocysts in the litter, originating from the vaccine or local wild-type strains. When re-infection cannot occur following vaccination, for instance when birds are housed in cages, protective immunity against challenge infections is not fully developed (Williams, 2002). The rate of transmission of vaccine and wild-type strains can affect the timing of exposure to vaccine oocysts in the flock cycle, the uniformity of uptake by individuals in the flock and the occurrence of re-infections, and consequently, affects the efficacy of interventions. Although many efficacy studies for different vaccination strategies have been carried out (Chapman et al., 2002; Williams, 2002), effects of vaccine intake by individual birds and subsequent effects on transmission of vaccine and wild-type strains, however, were not determined in these studies.

Vaccinating a large part of the flock at an early age, in theory, may thus be a very effective control strategy. In addition to applying live vaccine via the feed, water or by spraying the birds shortly after arrival in the poultry house, other application methods, including spraying the birds in the hatchery and using an edible gel in hatchery trays, were developed in an attempt to facilitate an early onset of immunity and uniformity of exposure to the vaccine. Furthermore, *in ovo* immunisation of embryonated eggs (Weber et al., 2004; Ding et al., 2005) might be an efficient method to induce an early and uniform exposure to vaccine, but is not yet applied on a large scale.

Vaccination and transmission
An alternative approach for facilitating early and uniform exposure to vaccinal oocysts might be to use the transmission capacity of the vaccine. Transmission characteristics of vaccine strains and factors that influence transmission of the vaccine and subsequent wild-type infections have not been determined previously, but could facilitate improvement of live vaccination strategies.

Transmission experiments were carried out to determine the rate of transmission of an *E. acervulina* vaccine strain (*chapter 6*), and to determine the level of protection against infection with a wild-type strain. The vaccine strain was transmitted quickly and the transmission rate parameter implied that, if this parameter could be extrapolated to the situation in the flock, 95% of the flock would become infected with vaccine oocysts within approximately five days after administration of the intended vaccine dose to 50% of the birds in a flock. Vaccinated and contact-vaccinated birds were equally protected against high oocyst output after challenge with the wild-type strain compared to unvaccinated birds. This suggests that mass application of a live vaccine in a broiler flock could potentially result in proper vaccination of the entire flock and protection against the adverse effects of a subsequent infection with wild-type strains.

Transmission of the wild-type strain was not significantly reduced after vaccination. This was unexpected, because the pair-wise study (*chapter 4*) showed that transmission was significantly reduced after priming. Apparently, the primary infection induced an immune response that was able to reduce both oocyst output and transmission between birds after secondary infection. The vaccine-induced immunity, however, was not able to significantly affect the probability of birds to become infected with a wild-type strain and only reduced oocyst output, and, also to a lesser degree than in the pair-wise experiment.

It is not clear why (contact)-vaccination was not as effective in protecting against the challenge infection, compared to the single high dose inoculation used in the pair-wise experiments. All birds in the vaccinated and contact-vaccinated groups had been shedding oocysts, which indicates that all birds in the group had successfully been "primed" with vaccine oocysts. Furthermore, multiple mild re-infections (that could occur during the vaccination experiment but not in the pair-wise experiment, where birds were kept on cages after the primary infection) have shown to be more effective in stimulating protective immune responses than single heavy infections (Joyner and Norton, 1976; Williams, 2002). Although it was shown that the live vaccine strain has the potential to reduce oocyst output and clinical signs in individual birds after challenge with wild-type strains (Williams, 1992), the immune response to a homologous strain might be more effective in reducing both oocyst output and transmission of infection. It remains unclear, however, which mechanisms are involved.

Nevertheless, it was shown that the vaccine strain was transmitted rapidly and induced equal levels of protection for vaccinated and contact-vaccinated birds, which might seem inconsistent with reports of outbreaks of clinical coccidiosis and poor performance in the field. It was shown that birds that are initially missed during initial vaccination do become contact-vaccinated, but this occurs later during the flock cycle compared to the directly vaccinated birds. This may result in a delay in build up of immunity. As discussed previously, a delay in protection of a substantial part of the flock may result in reduced production performance of the flock. This delay in exposure for the initially unvaccinated birds increases when the initial vaccine intake by chickens in the flock is lower.

Data on actual initial vaccine intake after mass application in a flock are, however, rare. In broiler flocks where one-day-old chicks were vaccinated using a course spray or were vaccinated before five days of age via drinking water, more than 90% of the birds were reported to shed oocysts within a week after vaccination (Schetters et al., 1999). The proportion of birds infected within six to eight days after eye spray vaccination was reported to vary between 86% and 95% of chicks (Chapman and Cherry, 1997). However, these results do not actually reflect the initial intake of the vaccine, as some spread of the *Eimeria* species with short prepatent periods already could have taken place. The transmission rate estimated from the experiments described in this thesis suggests that even when only 25% of the birds would initially be vaccinated, 95% of the flock would become contact-vaccinated within seven days after mass application. From an initial intake of 65% onwards this delay would be reduced to four days. The influence of initial vaccine intake on time of contact-vaccination, consequently, might be limited, but should be studied further. Experimental and field studies, aided with mathematical models, are necessary to study the effects of different application methods on initial vaccine intake, transmission, development of sufficient protective immunity and production performance of vaccinated flocks.

7.2.4. Reducing transmission to control coccidiosis

Prevention of introduction of the parasite into a flock
Transmission of an *Eimeria* infection between chickens occurs exclusively via the faecal-oral route, without an intermediate host or vector. The *Eimeria* species infecting chickens are strictly host-specific and so far no other avian species have been identified as potential host (McDougald, 2008). Coccidial oocysts are ubiquitous and highly resistant to disinfectants and environmental conditions. In soil, oocysts can survive for at least several weeks and up to nine months. In poultry litter, disintegration of oocysts starts after five days and viability of the oocyst population begins to wane after about three weeks, due to the presence of bacteria and ammonia (Reyna, 1983; Williams, 1995a).

Transmission of the parasite between pens, houses or even farms occurs mechanically, via faecal material, soil or dust from infected premises, mainly by movements of personnel or equipment and potentially by passive transfer via carriers such as rodents, earthworms, flies and beetles (Reyna et al., 1983; Belli et al., 2006). The large reproduction potential of *Eimeria* species and the high stocking densities and warm environment in commercial poultry houses are highly favourable for replication and accumulation of the parasite in the litter (Shirley et al., 2005). Consequently, high contamination levels occur in the poultry houses and their surroundings. Although disinfectants effective against *Eimeria*, such as ammonia and methyl bromide, are not applied due to their toxicity, removal of the litter, thorough cleaning and adopting a downtime period between flocks usually result in removal of most, but not all oocysts. Additional hygienic measures, such as poultry-house based clothing and footwear, reducing contact with visitors and equipment from other stables or farms, etcetera, can

further minimise the spread of oocysts (Graat et al., 1998). However, even in isolation facilities with stringent sanitation and quarantine procedures introductions of *Eimeria* have been reported (Reid, 1989). Infection of a new flock in litter-based farms is therefore considered to be practically unavoidable (Long, 1984; Reid, 1989; Williams, 1999; Allen and Fetterer, 2002; McDougald, 2008), especially because it is assumed that a single viable sporulated oocyst can infect a bird and can result in excretion of thousands of offspring that can induce a flock infection.

The experiments described in this thesis have shown that a dose of 5 sporulated oocysts could result in infection of the inoculated bird which subsequently resulted in extensive transmission to other chickens (*chapters 3 and 5*). This illustrates that a chain of infections can be initiated when only one bird ingests a low number of oocysts. Consequently, preventing a flock infection might be difficult as only a small number of oocysts can establish an infection in a single bird of the flock and a few oocysts are likely to remain in the poultry house, even after proper cleaning and disinfection.

However, although the introduction of *Eimeria* parasites in a flock might be difficult to prevent in the current situation, effective intervention strategies might eventually reduce the presence of oocysts in poultry houses, for example by affecting shedding patterns and reducing the amount of oocysts excreted by infected birds. This ultimately might result in a substantial reduction of the risk of infection of the first bird of the infection chain and consequently might significantly reduce transmission between flocks.

Reducing transmission within and between flocks
It is arguable whether or not aiming towards reducing transmission of the parasite is desirable for control of coccidiosis. It might be more advantageous to aim the efforts towards reducing the clinical signs and improving production performance in infected flocks, which has been the most important aim of control measures up to now. Furthermore, transmission of *Eimeria* species might even be beneficial, especially when it would result in exposure of many birds of the flock to the parasite at an early age, as this might induce protection against infections later during the flock cycle. However, as the efficacy of current strategies to control outbreaks and reduce economic losses is limited, novel approaches, including those aimed at reducing transmission are worth investigating.

Reducing transmission should either result in eradication of the wild-type parasite strains from a flock or at least in a reduction of transmission between flocks. When interventions reduce the between flock transmission to an extent where less than one flock becomes infected by a typical infected flock, eradication of the parasite from farms is feasible. However, whether this can be achieved and whether it can be a cost-effective and practical strategy is currently unknown.

Influencing the immune response is most likely an effective target for reducing transmission and perhaps eradication of the parasite. In *chapter 4* it was shown that acquired immunity can influence transmission which indicates that acquired immunity is not only

beneficial for protecting immune birds against clinical signs, but is also a potential effective target for substantially reducing transmission. In *chapter 6* it was shown that transmission of a wild-type strain was not reduced in vaccinated flocks. However, previous experimental results (*chapter 4*) with the wild-type strain suggest that perhaps a more homologous strain might induce a more effective protection against transmission. Furthermore, for other more immunogenic species, such as *Eimeria maxima*, vaccination might be more successful in reducing transmission. Nevertheless, oocyst output was significantly reduced after infection with the wild-type strain, even in birds that were not directly vaccinated but ingested the vaccine from the environment. Also, the oocyst load can perhaps be reduced increasingly after several successive rounds of vaccination, which might eventually reduce the transmission between flocks. Another possibility is that the oocyst population that eventually remains in the poultry house and the surroundings will consist mainly of the vaccine strains, which can have a reduced reproduction potential and pathogenicity (Williams, 1992; Shirley et al., 2005). If this would occur, this might have an influence on the transmission potential and clinical signs for subsequent flocks.

7.3. Future research

This thesis has revealed parts of the complex underlying processes of transmission dynamics of *Eimeria*. It was shown that not the oocyst load in the environment, but other factors, such as the probability of coming into contact with infectious faecal material, affect transmission of the parasite. Further research could be aimed towards studying the effects of distribution of infectious faecal material in a poultry house and behaviour of birds on the infection dynamics. Eventually, the factors that are associated with between flock transmission warrant further investigation. In addition, elucidating the factors that influence susceptibility (protective immunity) to invasion or multiplication of the parasite in host cells might also provide promising new outlooks on approaches to coccidiosis control.

Because the timing of exposure to vaccine oocysts in the flock cycle, the uniformity of uptake by individuals in the flock and the occurrence of re-infections can be influenced by transmission of vaccine and wild-type strains in a flock, studying factors that influence transmission might be important to improve current vaccination strategies. For comparisons of efficacy for different (application) strategies, the proportion of infectious birds and timing of infections of individual birds in the flock are important to evaluate. Moreover, factors that determine exposure to vaccine oocysts after application of the vaccine, that can influence transmission and re-infection and consequently the immune response, might be studied. When these factors are optimised, it could be studied whether the oocyst dose in commercial vaccines can be reduced, which would significantly increase cost-effectiveness and might stimulate more widespread use of vaccines in broilers.

In commercial flocks, other pathogens and environmental factors might affect the response of the host to *Eimeria* infections. Therefore, efficacy of interventions eventually should also be studied under field circumstances. When studying *Eimeria* in commercial flocks, different combinations of *Eimeria* species and combinations of vaccine and wild-type strains can be present simultaneously. Quantifying oocysts in faecal samples can be important for determining the time of vaccination/infection and the proportion of the flock that is vaccinated/infected. However, with the McMaster oocyst counting technique differentiation between species and strains is not possible. Furthermore, for large experimental or field studies the required sample sizes most likely exceed the number of samples that can be processed using this technique. A quantitative PCR can accurately determine oocyst output in samples of individual birds (*chapter 2*). Therefore, a multiplex PCR, that could quantify the number of oocysts for multiple species of *Eimeria* and could differentiate between vaccine and field strains, would therefore be very useful for further studies.

Combining field and experimental studies and mathematical models facilitates obtaining insight into the different aspects that determine infection dynamics. An integrative approach can eventually result in scenario studies that can be useful to quantify effects of interventions on the outcome of the disease and performance of the flock.

7.4. Conclusions of this thesis

This thesis has unravelled parts of the underlying mechanisms of an infection with *E. acervulina* in broiler flocks and has provided new perspectives on transmission dynamics that can be used for the improvement of current and development of novel approaches for the control of coccidiosis. The following main conclusions can be drawn from this thesis:

- The excreted dose of *E. acervulina* oocysts increases during successive generations of infection in a flock, but the transmission rate remains constant;
- Transmission of *E. acervulina* is not determined by the actual number of oocysts excreted with the faeces of infected birds, but most likely by the probability of chickens to come into contact with infectious faecal material;
- A primary infection with a wild-type *E. acervulina* strain can significantly reduce oocyst output and transmission between birds after a subsequent infection with a homologous strain;
- A live *E. acervulina* vaccine strain can be efficiently transmitted from vaccinated birds to contact-exposed birds in a flock and can induce an equal level of protection in vaccinated and contact-vaccinated chickens against high oocyst output after infection with an *E. acervulina* wild-type strain.

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Summary

Eimeria **infections in chicken flocks and the need for control**

One of the most common and expensive diseases in the poultry industry is coccidiosis, an intestinal disease caused by protozoan parasites of the genus *Eimeria*. In broilers, chickens raised for meat, infection with the most prevalent species, *Eimeria acervulina*, primarily results in diarrhoea, poor feed conversion and reduced body weight gain.

Eimeria species are ubiquitous and the infectious stage, the oocyst, is highly resistant to disinfectants and environmental conditions. After ingestion of sporulated oocysts, infected chickens excrete large numbers of oocysts in the litter, which can subsequently infect other birds in a flock. Furthermore, oocysts can be spread mechanically to other houses or farms by movements of personnel, equipment and vermin. Currently, introduction of infection is considered to be practically unavoidable and most broiler flocks are given anticoccidial drugs in the feed to reduce symptoms and production losses. However, increasing drug resistance, public concerns about residues in poultry products and governmental bans against prophylactic use of anticoccidials have increased demands for alternative control strategies, such as vaccination.

The development and improvement of control strategies requires a thorough comprehension of the factors that influence the course of infection in a flock. Parasite characteristics, the infectivity and susceptibility of individual birds, environmental factors and behaviour of birds all interact and mutually influence each other. This complex interplay determines the infection dynamics of *Eimeria* species, and consequently, the clinical outcome and production performance for the flock. An increased understanding of the underlying mechanisms that determine these infection dynamics can facilitate control efforts.

Although many aspects of *Eimeria* infections have already been described, most studies up to now have mainly focused on interactions between the parasite and the individual host, but have not incorporated the dynamical aspects of the infection in the population, such as transmission characteristics. In this thesis, transmission experiments were carried out to obtain insights into the relative contribution of different factors to the transmission dynamics of *E. acervulina* in broilers.

Oocyst excretion and transmission

A crucial aspect when studying transmission of an infectious agent is determining for individual chickens in a group if and when each bird becomes infected. Furthermore, the number of excreted oocysts can provide an indication of the severity of the infection and the contribution to the oocyst load in the environment. Consequently, a method had to be developed to accurately determine the presence and level of excreted *E. acervulina* oocysts in faeces of chickens in a group. In *chapter 2* it was shown that McMaster oocyst counts of single individual faecal droppings, collected by placing birds in a temporary individual cage for

one or two hours, provided reliable estimates of oocyst output compared to the gold standard of oocyst counts of 24 h faecal collections. The "single dropping" procedure was therefore used to identify infection status and determine oocyst output for individual birds in the subsequent transmission experiments. Furthermore, it was shown that a quantitative PCR on cloacal swabs accurately reflected oocyst output. In contrast with McMaster oocyst counts, this technique allows for large sample sizes and differentiation between *Eimeria* species and vaccine and wild-type strains, which might be useful for future field studies.

Transmission experiments with a pair-wise design, where one inoculated and one contact-exposed bird are housed together, were carried out to study the relation between oocyst dose and transmission (*chapter 3*). It was shown that even a low dose of 5 sporulated *E. acervulina* oocysts, administered to the inoculated bird, could start a "chain of infections", as the contact-exposed bird in each pair became infected. Furthermore, although a higher inoculation dose resulted in higher oocyst output by the inoculated birds, the transmission rate was not significantly influenced by the inoculation dose. Contact-infected chickens shed significantly more oocysts than their inoculated pen mates, which indicated that for successive generations of infection the oocyst output can increase.

The level of oocyst output and effects on transmission for successive generations of infection were studied by carrying out transmission experiments with groups of broilers (*chapter 5*). In these experiments one bird was inoculated with a low dose of 5 sporulated *E. acervulina* oocysts and 19 birds were contact-exposed. In agreement with the pair-wise study, it was shown that the oocyst output increased for successive generations, but the transmission rate remained constant. The results of the pair-wise and group experiments provided a novel outlook on transmission dynamics: the transmission rate is most likely determined by the probability of birds to come into contact with infectious faecal material in the environment, irrespective of the actual number of oocysts excreted in the faeces. Transmission dynamics, therefore, might be determined by the degree and dispersal of faecal contamination in the environment, which can be associated with factors such as the volume of the excreted droppings, distribution of faeces through a poultry house, dispersal and survival of oocysts in the different layers of the litter, litter pecking behaviour of birds, movements of birds through the house, etcetera. These factors might be potential targets for the control of coccidiosis and therefore the influence of these factors on the infection dynamics should be studied further.

A theory that may explain a potential underlying process of transmission dynamics was introduced in *chapter 5* and was based on comparisons of the results of the pair-wise and group experiments and simulation models. It was suggested that, especially at the start of a flock infection when only a few chickens are excreting contaminated faeces, the transmission rate might gradually increase over a few days to a plateau level, at which it remains constant for as long as the number of infectious individuals remains constant. This build up of infectivity should be considered when analysing the data of future transmission experiments.

Immunity and transmission

In (partially) immune birds, the development of the parasite in intestinal cells is inhibited, and consequently, clinical signs and oocyst output are reduced after re-infection with a homologous species. The level of protection against coccidiosis can depend on the *Eimeria* species or strain, nutritional status, genetic makeup of the host and on the level and duration of the exposure to oocysts, i.e. the infection history of the hosts. The infection history of individual chickens, therefore, is determined by and conversely affects the infection dynamics of *Eimeria* infections in a flock.

A pair-wise experiment was carried out with different combinations of previously infected (primed) chickens to determine effects of an infection history on oocyst output, immune parameters and transmission after an infection with a homologous wild-type *E. acervulina* strain (*chapter 4*). When both the inoculated and contact bird had been primed, transmission was significantly reduced and oocyst output was hardly detectable. This study demonstrated that immunity can have a considerable impact on infection dynamics and might be an important target for the control of coccidiosis.

One of the most promising alternatives to anticoccidial drugs, therefore, is vaccination. Live vaccines are frequently used in layer and breeder flocks. The use in broilers remains limited, due to relatively high costs of the vaccine in relation to the small profit margins. Furthermore, there are concerns about the timely onset of protective immunity in birds with such a short life span and fears for adverse effects on growth. When vaccination strategies can be optimised, this might stimulate the use of vaccines in the broiler sector. It has often been suggested that crucial aspects for efficacy of live vaccination are the uniformity of uptake of the intended oocysts dose by a large proportion of the flock, which might not be achieved during mass application, and subsequent re-infections that can boost immunity. The rate of transmission of vaccine and wild-type strains can affect the timing of exposure to (vaccine) oocysts in the flock cycle, the uniformity of uptake by individuals in the flock and the occurrence of re-infections, and consequently, can affect the efficacy of interventions.

Transmission experiments were carried out to study the transmission dynamics of vaccine and subsequent wild-type strains in groups of broilers (*chapter 6*). The transmission rate of an *E. acervulina* vaccine strain from vaccinated to unvaccinated contact-exposed birds was determined and the level of protection between these vaccinated and contact-vaccinated birds against a subsequent infection with an *E. acervulina* wild-type strain was compared. The vaccine strain was transmitted to all contact-exposed birds and the oocyst output after the challenge infection was equally reduced for vaccinated and contact-vaccinated birds, compared to unvaccinated birds. However, transmission of the subsequent wild-type strain was not significantly reduced in the vaccinated and contact-vaccinated groups compared to the unvaccinated groups. This result was remarkable, because during the pair-wise study not only the oocyst output, but also transmission, was significantly reduced when inoculated and contact-exposed birds had received a primary infection. Apparently, the immune response

induced by the vaccine was able to reduce multiplication of the parasite, but did not reduce the probability of birds to become infected with the wild-type strain parasites. Possibly, protective immunity against the wild-type strain is more effective when induced by the same wild-type strain, than when induced by a vaccine strain. However, whether this is true and which mechanisms might be involved remains unclear and requires further research.

Nevertheless, the transmission rate parameter implied that, if this parameter could be directly extrapolated to the situation in a commercial flock, 95% of a flock would become infected with vaccine oocysts within approximately five days after administration of the intended vaccine dose to 50% of the birds. Because live vaccines can spread efficiently through the whole flock after mass application and can provide protection against the adverse effects of a subsequent infection, one might wonder why outbreaks of disease and production performance are feared and reported in vaccinated broiler flocks. Although the vaccine is transmitted from vaccinated to unvaccinated chickens, contact-vaccination occurs at least four days later and this delay in vaccine exposure increases when the initial vaccine intake of the chickens in the flock is lower. When exposure to (vaccine) oocysts has not yet resulted in sufficient protective immunity for a substantial part of the flock during the peak of oocyst load in the litter, usually around the fifth week of the flock cycle, clinical coccidiosis and poor flock performance can occur. Possibly, a small delay in exposure to vaccine oocysts may hamper the immune system in its race against the increasing oocyst load in the environment. Furthermore, a delay in exposure may also lead to a shorter time for compensatory growth after infection with vaccine or wild-type oocysts before slaughter, which might further reduce production revenues. Experimental and field studies, aided with mathematical models, to study the effects of different application methods on initial vaccine intake, transmission and production performance are necessary to further elucidate the factors involved and might help to improve efficacy of vaccination strategies.

The main conclusions of this thesis

This thesis has unravelled parts of the underlying mechanisms of an infection with *E. acervulina* in broiler flocks and has provided new perspectives on transmission dynamics that may facilitate improvement of current and development of novel approaches for the control of coccidiosis. The following main conclusions can be drawn from this thesis:

- The excreted dose of *E. acervulina* oocysts increases during successive generations of infection in a flock, but the transmission rate remains constant;
- Transmission of *E. acervulina* is not determined by the actual number of oocysts excreted with the faeces of infected birds, but most likely by the probability of chickens to come into contact with infectious faecal material;

- A primary infection with a wild-type *E. acervulina* strain can significantly reduce oocyst output and transmission between birds after a subsequent infection with a homologous strain;
- A live *E. acervulina* vaccine strain can be efficiently transmitted from vaccinated birds to contact-exposed birds in a flock and can induce an equal level of protection in vaccinated and contact-vaccinated chickens against high oocyst output after infection with an *E. acervulina* wild-type strain.

Samenvatting

Eimeria infecties in koppels kippen en de noodzaak voor bestrijding

Eén van de meest voorkomende en kostbare ziekten in de pluimvee-industrie is coccidiose, een darmziekte veroorzaakt door protozoaire parasieten van het geslacht _Eimeria_. Bij vleeskuikens, kippen die voor de vleesproductie worden gehouden, resulteert een infectie met de meest voorkomende soort, _Eimeria acervulina_, voornamelijk in diarree, hogere voederconversies en groeiremming.

    Eimeria soorten komen ubiquitair voor en het infectieuze stadium van de parasiet, de oöcyste, is zeer resistent tegen desinfecterende middelen en omgevingsfactoren. Na orale opname van gesporuleerde oöcysten scheiden geïnfecteerde dieren grote aantallen oöcysten uit in het strooisel, die vervolgens andere dieren in het koppel kunnen besmetten. Daarnaast kunnen oöcysten mechanisch worden verspreid naar andere stallen of bedrijven door verplaatsingen van personeel, materieel en ongedierte. Tegenwoordig wordt het binnenslepen van de infectie als onvermijdelijk beschouwd en de meeste koppels vleeskuikens krijgen coccidiostatische diergeneesmiddelen via het voer om de symptomen en productieverliezen te beperken. Echter, door de toenemende resistentie tegen coccidiostatica, de bezorgdheid van de consument over residuen in producten van dierlijke oorsprong en wettelijke beperkingen aan het preventief gebruik van middelen tegen coccidiose, neemt de behoefte aan alternatieve bestrijdingsstrategieën, zoals vaccinatie, toe.

    De ontwikkeling en verbetering van bestrijdingsstrategieën voor coccidiose vereisen een grondig begrip van de factoren die het verloop van een infectie beïnvloeden in een koppel. De eigenschappen van de parasiet, de infectiviteit en gevoeligheid van individuele dieren, omgevingsfactoren en het gedrag van de kippen hebben onderling verband met elkaar en beïnvloeden elkaar. Dit complexe samenspel bepaalt de infectiedynamiek van _Eimeria_ soorten en daarmee de klinische effecten en de productieprestaties voor het koppel. Een beter begrip van de onderliggende mechanismen die deze infectiedynamiek bepalen kan ten goede komen aan de bestrijding van coccidiose.

    Hoewel veel aspecten van _Eimeria_ infecties al zijn beschreven hebben de meeste studies vooral de nadruk gelegd op interacties tussen de parasiet en de individuele gastheer, maar zijn de dynamische aspecten van de infectie in de populatie, zoals transmissiekenmerken, niet meegenomen. Voor dit proefschrift zijn transmissie-experimenten uitgevoerd om inzicht te krijgen in de relatieve bijdrage van verschillende factoren aan de dynamiek van de overdracht van infecties, de transmissiedynamiek, van _E. acervulina_ bij vleeskuikens.

Oöcystenuitscheiding en transmissie

Een cruciaal aspect bij de bestudering van transmissie van een besmettelijk agens is het bepalen voor individuele dieren in een groep of en wanneer een dier geïnfecteerd raakt. Bovendien kan het aantal uitgescheiden oöcysten een indicatie geven van de ernst van de

infectie en van de bijdrage aan de infectiedruk met oöcysten in de omgeving. Daarom was het noodzakelijk om een methode te ontwikkelen voor het nauwkeurig vaststellen van de aanwezigheid en het aantal uitgescheiden *E. acervulina* oöcysten in de mest van individuele kippen in een groep. In *hoofdstuk 2* werd aangetoond dat McMaster oöcystentellingen van individuele mestmonsters ("single droppings"), verzameld na het tijdelijk apart plaatsen van individuele kippen gedurende één of twee uur, een betrouwbare meting geeft van de uitscheiding van oöcysten. Hiertoe werden de uitkomsten vergeleken met de gouden standaard methode, namelijk het kwantificeren van het aantal oöcysten in mest dat is geproduceerd gedurende 24 uur. De "single dropping" procedure werd vervolgens gebruikt om de infectiestatus en de mate van uitscheiding in individuele dieren te bepalen tijdens de transmissie-experimenten. Daarnaast werd aangetoond dat een kwantitatieve PCR op cloacaswabs ook gebruikt kan worden voor het bepalen van het aantal oöcysten. In tegenstelling tot de McMaster oöcystentellingen kunnen bij een PCR grotere aantallen monsters verwerkt worden en kunnen de verschillende *Eimeria* soorten en vaccin en wild-type stammen van elkaar onderscheiden worden, wat noodzakelijk kan zijn voor toekomstige veldexperimenten.

Transmissie-experimenten met een tweetal dieren ("paartjesexperimenten"), waarbij één geïnoculeerd dier samen met één contactdier in hetzelfde hok wordt gehuisvest, werden uitgevoerd om de relatie tussen oöcystendosis en transmissie te bestuderen (*hoofdstuk 3*). Er werd aangetoond dat zelfs een lage dosis van 5 gesporuleerde *E. acervulina* oöcysten, toegediend aan het geïnoculeerde dier, een "keten van infecties" kon starten, want het contactdier van elk paartje raakte besmet. Hoewel een hogere inoculatiedosis resulteerde in een hogere oöcystenuitscheiding door de geïnoculeerde dieren werd de transmissiesnelheid niet significant beïnvloed door de inoculatiedosis. Contactgeïnfecteerde kippen vertoonden een signficant hogere uitscheiding dan hun geïnoculeerde hokgenoten, wat aangeeft dat de mate van oöcystenuitscheiding voor opeenvolgende generaties van de infectie kan toenemen.

De mate van uitscheiding en de effecten op transmissie voor opeenvolgende generaties tijdens een koppelinfectie werden verder bestudeerd door het uitvoeren van transmissie-experimenten in groepen van vleeskuikens (*hoofdstuk 5*). In deze experimenten werd één kuiken geïnoculeerd met een lage dosis van 5 gesporuleerde *E. acervulina* oöcysten en samen gehouden met 19 contactdieren. Net als in de paartjesexperimenten werd aangetoond dat de oöcystenuitscheiding toenam voor opeenvolgende generaties, maar dat de transmissiesnelheid constant bleef. De resultaten van de paartjesexperimenten en groepsexperimenten hebben een nieuw inzicht gegenereerd in de transmissiedynamiek: de transmissiesnelheid wordt hoogst waarschijnlijk bepaald door de kans van de dieren om in contact te komen met besmettelijke mestdeeltjes in het milieu, ongeacht het aantal oöcysten dat wordt uitgescheiden in de mest. De transmissie van een infectie in een koppel zou dus kunnen worden bepaald door de mate en verspreiding van fecaal materiaal in de omgeving, wat gerelateerd kan zijn aan diverse factoren, zoals het volume van de mest, de verspreiding

van de mest door de stal, verspreiding en overleving van oöcysten in de verschillende lagen van het strooisel, pikgedrag naar het strooisel door de dieren, bewegingen van de kippen door de stal, etcetera. Deze factoren kunnen potentiële aangrijpingspunten zijn voor bestrijdingsmaatregelen en daarom is het nuttig om de invloed van deze factoren op de infectiedynamiek verder te bestuderen.

In *hoofdstuk 5* werd een theorie beschreven dat mogelijk een onderliggend proces van de transmissiedynamiek kan verklaren. Deze theorie was gebaseerd op vergelijkingen van resultaten van paartjes- en groepsexperimenten en simulatiemodellen. Er werd geponeerd dat, met name aan het begin van een koppelinfectie wanneer slechts een klein aantal kippen infectieuze mest uitscheidt, de transmissiesnelheid geleidelijk toeneemt over een aantal dagen tot een plateau niveau, waarna het constant blijft zolang het aantal infectieuze individuen constant blijft. Bij het analyseren van toekomstige transmissie-experimenten moet rekening gehouden worden met deze opbouw van infectiviteit.

Immuniteit en transmissie

In (gedeeltelijk) immune kippen wordt de ontwikkeling van de parasiet in darmcellen geremd en hiermee worden de klinische symptomen en uitscheiding van oöcysten verminderd na herinfectie met een homologe soort. De mate van bescherming tegen coccidiose kan afhangen van de *Eimeria* soort of stam, voedingstoestand, genetische opbouw van de gastheer en van de duur en mate van blootstelling aan oöcysten, met andere woorden de infectiegeschiedenis van de gastheer. De infectiegeschiedenis van individuele kippen wordt derhalve bepaald door, en beïnvloedt op zijn beurt de infectiedynamiek van *Eimeria* infecties in een koppel.

Een paartjesexperiment werd uitgevoerd met verschillende combinaties van eerder besmette kippen om de effecten van een infectiegeschiedenis op uitscheiding van oöcysten, immuunparameters en de transmissie te bepalen na een infectie met een homologe wild-type *E. acervulina* stam (*hoofdstuk 4*). Wanneer zowel het geïnoculeerde als het contactdier een eerdere infectie hadden gehad, werd de transmissie aanzienlijk gereduceerd en was uitscheiding van oöcysten nauwelijks waarneembaar na herinfectie. Deze studie toonde aan dat de immuniteit een aanzienlijke invloed kan hebben op de infectiedynamiek en daarom een belangrijk aangrijpingspunt kan zijn voor de bestrijding van coccidiose.

Vaccinatie is derhalve één van de meest veelbelovende alternatieven voor gemedicineerd voer. Levende vaccins worden regelmatig gebruikt bij leg- en vermeerderingsdieren. Het gebruik bij vleeskuikens is vooralsnog beperkt, als gevolg van de relatief hoge kosten van het vaccin in relatie tot de kleine winstmarges. Verder vraagt men zich vaak af of de dieren voldoende snel beschermende immuniteit ontwikkelen, gezien de korte levensduur, en vreest men voor negatieve effecten op de groei. Wanneer vaccinatiestrategieën worden geoptimaliseerd zou dit het gebruik van vaccins in de vleeskuikensector kunnen stimuleren. Er

wordt vaak gesuggereerd dat de uniformiteit van de opname van de beoogde oöcystendosis door een groot deel van het koppel, wat wellicht niet altijd goed lukt bij een massa-applicatie, en immuniteitstimulerende herinfecties na vaccinatie, cruciaal zijn voor de effectiviteit van de vaccinatie. De snelheid van transmissie van vaccin en wild-type stammen kan invloed hebben op het moment waarop dieren worden blootgesteld aan (vaccin) oöcysten tijdens de productieronde, de uniformiteit van de opname van oöcysten door dieren in het koppel en het optreden van herinfecties en kan dus een grote invloed hebben op de effectiviteit van bestrijdingsmaatregelen.

Transmissie-experimenten werden uitgevoerd om de transmissiedynamiek van het vaccin en van de hiernavolgende wild-type stammen in groepen vleeskuikens te bestuderen (*hoofdstuk 6*). De transmissiesnelheid van een *E. acervulina* vaccinstam van gevaccineerde naar niet-gevaccineerde contactdieren werd bepaald en de mate van bescherming van gevaccineerde en contactgevaccineerde kippen tegen een infectie met een *E. acervulina* wild-type stam werden vergeleken. De vaccinstam spreidde naar alle contactdieren en de oöcystenuitscheiding na de wild-type infectie was aanzienlijk lager voor zowel gevaccineerde- als contactgevaccineerde dieren in vergelijking met niet-gevaccineerde dieren. Echter, de transmissiesnelheid van de wild-type stam was niet significant verlaagd in de gevaccineerde en contactgevaccineerde groepen, in vergelijking met de niet-gevaccineerde groepen. Dit resultaat was opmerkelijk, want tijdens paartjesexperimenten was niet alleen de uitscheiding, maar was ook de transmissie significant verminderd wanneer het geïnoculeerde dier en contactdier beiden een eerdere infectie hadden gehad. Blijkbaar was de door de vaccinstam opgewekte immuunrespons wel in staat om de vermeerdering van de parasiet te beperken, maar niet om de kans op besmetting met de wild-type stam te verminderen. Wellicht is de immuniteit tegen de wild-type stam effectiever wanneer deze is opgewekt door een identieke wild-type stam, dan wanneer opgewekt door een vaccinstam. Echter, of dit het geval is en welke mechanismen hierbij een rol zouden kunnen spelen is nog onduidelijk en dient nader onderzocht te worden.

Desalniettemin, wanneer de transmissiesnelheid gemeten tijdens deze experimenten geëxtrapoleerd zou kunnen worden naar de situatie in een commercieel koppel, zou 95% van een koppel, binnen ongeveer vijf dagen na toediening van de beoogde dosis vaccin aan 50% van de kippen, kunnen worden besmet met vaccinoöcysten. Aangezien levende vaccins efficiënt kunnen spreiden door het gehele koppel na de massa-applicatie en bescherming kunnen bieden tegen de nadelige gevolgen van een latere wild-type infectie, vraagt men zich wellicht af waarom uitbraken van de ziekte en productieverliezen worden gevreesd en gerapporteerd in gevaccineerde koppels vleeskuikens. Hoewel transmissie optreedt van de gevaccineerde- naar de niet-gevaccineerde kippen, vindt deze contactvaccinatie ten minste vier dagen later plaats. Deze vertraging in de blootstelling neemt verder toe wanneer minder dieren oöcysten opnemen tijdens de oorspronkelijke vaccinatie. Wanneer de blootstelling aan (vaccin) oöcysten nog niet geresulteerd heeft in voldoende beschermende immuniteit voor een substantieel deel van het koppel op het moment dat de infectiedruk in het strooisel een

hoogtepunt bereikt, wat meestal rond de vijfde week van de ronde optreedt, kunnen klinische coccidiose en slechte prestaties van het koppel optreden. Mogelijk kan een kleine vertraging in de blootstelling aan vaccinoöcysten ervoor zorgen dat het immuunsysteem de race tegen de toenemende infectiedruk in de omgeving verliest. Bovendien kan een vertraging in de blootstelling ook leiden tot een kortere tijd voor compensatoire groei na infectie met vaccin- of wild-type oöcysten voordat de dieren geslacht worden, wat kan zorgen voor een verdere afname in de productie-inkomsten. Om de effecten van verschillende applicatiemethoden op de eerste vaccinopname, transmissie en productieprestaties verder te bestuderen zijn experimentele- en veldstudies nodig, aangevuld met wiskundige modellen, om verder te ontrafelen welke factoren betrokken zijn bij deze processen. Met deze kennis kan uiteindelijk de effectiviteit van vaccinatiestrategieën verbeterd worden.

De belangrijkste conclusies van dit proefschrift

Dit proefschrift heeft enkele onderliggende mechanismen van een infectie met *E. acervulina* in koppels vleeskuikens ontrafeld en heeft nieuwe perspectieven op de transmissiedynamiek geopend die gebruikt kunnen worden voor het verbeteren van huidige en het ontwikkelen van nieuwe benaderingen in de bestrijding van coccidiose. De meest belangrijke conclusies die getrokken kunnen worden uit dit proefschrift zijn:

- De uitgescheiden dosis van *E. acervulina* oöcysten neemt toe tijdens opeenvolgende generaties van een koppelinfectie, maar de transmissiesnelheid blijft constant;
- Transmissie van *E. acervulina* wordt niet bepaald door het aantal oöcysten dat wordt uitgescheiden met de mest van geïnfecteerde dieren, maar waarschijnlijk door de kans om in aanraking te komen met infectieus fecaal materiaal;
- Een primaire infectie met een wild-type *E. acervulina* stam kan de oöcystenuitscheiding en transmissie tussen kippen aanzienlijk verlagen na een herinfectie met een homologe stam;
- Een levende *E. acervulina* vaccinstam kan efficiënt spreiden van gevaccineerde dieren naar contactdieren in een koppel en kan een even hoge mate van bescherming induceren in gevaccineerde en contactgevaccineerde kippen tegen hoge uitscheiding na infectie met een *E. acervulina* wild-type stam.

Dankwoord

Veel mensen hebben mij tijdens dit promotieonderzoek bijgestaan op verschillende manieren.

Allereerst ben ik mijn begeleidingsgroep erg dankbaar voor de begeleiding tijdens dit project. Bij Annemarie kon ik altijd mijn ei kwijt, zowel op het gebied van onderzoek als op het persoonlijke vlak. Naast dat ze haar taken als co-promotor uitstekend vervulde (snel reageren op vragen en stukken, altijd bereid te brainstormen) ging ze nog vele stapjes verder. Zij kon mij motiveren wanneer het project moeizaam verliep en leerde me dat het belangrijk was positief in het project te blijven staan en de bevindingen met veel zelfvertrouwen uit te dragen. Het lukte haar altijd mijn langdradige stukken naar een acceptabel aantal woorden te krijgen en haar relativerende opmerkingen ("nou, en?") motiveerden mij om beter te formuleren waarom de bevindingen zo interessant waren. In de stal en het lab brachten we de werkefficiëntie naar ongekende hoogten, terwijl we ondertussen heerlijk konden kletsen en lachen. Annemarie, bedankt voor alle energie die je in dit project hebt gestoken, voor al je hulp bij de experimenten in de weekenden en voor alle peptalks. Hopelijk zullen we ook in de toekomst vaak samenwerken. Mijn promotoren, Arjan Stegeman en Mart de Jong, ben ik zeer dankbaar voor hun inzet, vertrouwen en voor alles wat ik heb geleerd. Ze hebben veel energie gestoken in het brainstormen over proefplannen en het lezen van allerlei versies van artikelen. Ondanks diverse afwijzingen van artikelen bleven ze geloven in het project en hun enthousiasme hielp mij om de motivatie op peil te houden. Arjan heeft me daarnaast erg geholpen bij het bewaken van de verdeling van mijn tijd over onderwijs en onderzoek. Mart heeft vele uurtjes met mij achter de computer doorgebracht. Het moet een enorme uitdaging geweest zijn om de wiskundige modellen uit te leggen aan een dierenarts, maar dit werd altijd met veel enthousiasme en geduld gedaan. Ook heb ik de steun en het begrip van Arjan en Mart op momenten dat ik het moeilijk had erg gewaardeerd.

Vooral bij de start van mijn werk als pluimveedierenarts/onderzoeker heeft Jo van Eck, het toenmalige hoofd van de afdeling Pluimvee, mij veel waardevolle (levens)lessen meegegeven. Tijdens het monotraject pluimvee gaf hij mij de ruimte om met hem de eerste stappen op het gebied van pluimveeonderzoek te doen. De experimenten in het Spelderholt (een dependance van GD Deventer in Beekbergen) waren altijd een echt feest, met veel ruimte voor grappen, grollen, goede gesprekken en daarnaast hard werken. Ook hielp hij mij regelmatig in het weekend tijdens de experimenten in Wageningen. Kippen wegen, bloed- en mestmonsters verzamelen, inbrengen van wattenstaafje in cloaca's, overal was hij voor te porren. Jo gooide mij al snel in het diepe voor wat betreft het onderwijs en bedrijfsbezoeken. Daarvan heb ik veel geleerd en zijn vertrouwen in mij als docent/dierenarts heb ik erg gewaardeerd. Het door hem geïnitieerde en voor mij op maat gemaakte traject voor Nederlands specialist Pluimveegezondheidszorg heeft mij in staat gesteld veel te leren en zal hopelijk resulteren in honorering van mijn aanvraag voor Europees specialist.

Mijn paranimfen, Wil Landman en Herman Peek, hebben ook vanaf het begin een essentiële rol gespeeld. Met Wil heb ik met veel plezier gewerkt aan vele projecten. Van zijn allereerste les "orde en netheid op het bureau (en in je hoofd) en goed archiveren van

documenten in de computer", heb ik nog dagelijks veel profijt. Daarnaast hebben onze fijne gesprekken mij veel inzicht gegeven in mijn persoonlijke ontwikkeling. Herman heeft mij alle praktische kneepjes van het coccidiosewerk bijgebracht. Met veel plezier heb ik met hem gewerkt tijdens diverse experimenten. Ook kon ik altijd bij hem terecht met mijn vragen en fungeerde hij als wandelende bibliotheek. Tijdens het congres in Brazilië hebben we veel kunnen brainstormen over het werk en het leven in het algemeen en daarnaast ook erg veel lol met elkaar gehad, samen met Wil en Willem Swinkels. Bedankt dat jullie de rol van paranimf zo enthousiast hebben aanvaard en vervuld.

In het begin van mijn promotietraject was ik regelmatig werkzaam in Wageningen. Tijdens en rondom experimenten heb ik prettig samengewerkt met veel mensen, waaronder Lisette Graat (bedankt voor je begeleiding in de eerste jaren en hulp tijdens experimenten bij nacht en ontij), Klaas Frankena, Lora van der Kleijn en Nanette van Hapert (bedankt voor het regelen van vele zaken), de proefdierverzorgers en coördinatoren (waaronder Ries Verkerk en Roel Terluin), Aart Lammers, Mike Nieuwland, Ger de Vries Reilingh, Ariëtte van Knegsel, Martijn Bouwknegt, Marijke Schouten, Liesbeth Mollema, Ellen van Eerden en vele anderen.

De experimenten die ik heb gedaan waren veelal erg arbeidsintensief, maar door de hulp van velen was het toch mogelijk het werk gedaan te krijgen. Veel studenten (inmiddels allang afgestudeerd) hebben hierbij een belangrijke rol gespeeld, waaronder Merlijn Kense, Rudy Cornelissen, Niels Hannink, Tariq Halasa, Ewaldus Wera, en vele anderen. Diverse onderzoekstagiaires hebben mij erg intensief bijgestaan gedurende vele maanden: Christiaan ter Veen, Chantal Schoenmaker, Robin Zijlmans en Huiming Liu. Het was erg prettig om met jullie samen te werken. De dierverzorgers van Wageningen (hierboven al genoemd) en in Utrecht hebben zowel de kuikens als mij uitstekend bijgestaan tijdens de experimenten. Freek Weites heeft tevens een grote rol gespeeld bij voorbereidingen van experimenten, door veel te regelen op het gebied van huisvesting en logistiek. Freek, je werktempo en hart voor de zaak zijn bewonderenswaardig en bovendien maakte je me altijd erg vrolijk! Daarnaast hebben Arie Plomp, Tijmen den Ouden, Hans Lutz en vele anderen een belangrijke rol gespeeld bij de (coördinatie van) de dierverzorging. De ondersteuning van Halco (Frans van Kooi, Maurits, Nordine en Jamal) heb ik ook enorm gewaardeerd. Verder wil ik de coördinatoren (Helma Avezaat, Kees Brandt) en biotechnisch laboranten bij het GDL (Elise, Kiki, Gerard, en anderen) bedanken voor de zeer prettige en professionele wijze waarop de experimenten daar zijn uitgevoerd. Ook de biotechnisch laboranten van de proefdierfaciliteit van Deventer (Johan Welner, Machiel Esmann, Ad Klein Koerkamp) hebben me op verschillende manieren bijgestaan, door het goed verzorgen van de eieren/eendagskuikens, hun adviezen en de gezelligheid wanneer ik daar aan het werk was.

De dames "aan de overkant" van het reproductielab, Leni van Tol, Ineke Daemen en Elly Zeinstra: hartelijk dank voor het mogen gebruiken van labmaterialen en jullie hulp bij het plaatsen van oöcysten in capsules. Ineke was daarnaast een talent voor het tellen van oöcysten en mijn steun en toeverlaat tijdens het labwerk en de invoer van data tijdens één van de experimenten. Het was heerlijk om met je samen te werken, Ineke!

De samenwerking met de Animal Sciences Group (Willem Swinkels, Annemarie Rebel, Wim Boersma, Jan Cornelissen) was ook erg prettig en heeft een mooi artikel opgeleverd. Hans Heesterbeek verdient ook veel lof voor het opstarten van dit project. Hij heeft mij warm gemaakt voor dit project en mij naar voren geschoven als kandidaat voor deze AIO positie. Het was jammer dat je een stap terug moest doen uit mijn begeleidingsgroep, maar het was erg prettig om met je samen te werken. Dankzij de hulp van Damer Blake van het Institute for Animal Health in Compton in Engeland, hebben we gebruik kunnen maken van een qPCR. Thank you, Damer, for all your work on the manuscript in Chapter 2. I learned a lot and enjoyed working with you and the other people in the lab during my visit to Compton. And it was nice to be included as a participant in your annual running contest! Voor het laatste experiment ben ik tevens veel dank verschuldigd aan Intervet/Schering-Plough Animal Health voor het aanleveren van vaccinstammen. Prof Schetters en Hay Janssen hebben hier veel tijd en energie in gestoken en ons bovendien erg prettig ontvangen toen wij de stammen kwamen halen. Hartelijk dank en hopelijk kunnen we in de toekomst nog verder samenwerken. Uiteraard wil ik ook de leescommisie bedanken voor het beoordelen van dit proefschrift.

Ik ben de pluimveecollega's buitengewoon dankbaar voor de steun en bereidheid om zoveel werk op te vangen in drukke perioden. Mieke, bedankt dat je zo enorm je best hebt gedaan om mij uit de wind te houden en voor je bezorgdheid en medeleven. Marius, ik heb enorm veel van je geleerd en heb veel respect voor je positiviteit, tomeloze energie en hart voor de zaak. Franny, je bent een enorm fijne collega en vriendin. Bedankt dat je altijd voor me klaarstaat en met me meedenkt. Ook Jan, Anita, Ellen, Twan, Peter en Jos Heijmans: bedankt voor jullie steun, de prettige samenwerking en het opvangen van werkzaamheden. Verder wil ik alle collega's van de pluimveeafdeling van GD Deventer bedanken: bedankt voor alles wat ik van jullie heb geleerd en de fijne samenwerking. Met veel (oud)medewerkers van de afdeling Landbouwhuisdieren heb ik erg leuk samengewerkt. Don Klinkenberg en Hans Vernooij hebben vele tijd gestoken in hun hulp bij de wiskundige/ statistische analyses: hartelijk dank hiervoor. En andere mensen die ik toch met nadruk nog wil noemen omdat ze een grote bijdrage leveren aan mijn werkplezier zijn Wikke, Jacomijn, Tijs, Arie, Leo en de rest van de varkentjes, Frank, Annet en de andere dames van het secretariaat, Joyce, Nienke, Maite en de overige epidemiologen, Lonneke Vervelde, Jaap Wagenaar en oud medewerksters Marian Bos, Ekelijn Thomas, Marieke Augustijn en Marieke Berkeveld.

Het is vaak niet goed in woorden uit te drukken wat mensen voor je betekenen en welke rol ze hebben gespeeld en dat geldt vooral voor mensen in mijn privéleven. Mijn moeder die ik al vele jaren moet missen, mijn vader Jan (erg fijn dat je mooie tekeningen hebt geleverd voor de kaft), zus Patricia en nichtjes Carmen en Celeste, de rest van de familie, de "schoonfamilie", Brian Klink, mijn vrienden (Anja en Bryan, Ruud en Diana, Else: bedankt voor jullie steun en begrip): jullie hebben allemaal een warme plek in mijn hart. Lieve Teunis, je hebt enorm veel te stellen met mij, onder andere door alle tijd en energie die in dit proefschrift is verdwenen. Ik heb daarnaast enorm veel van je geleerd. Bedankt voor je steun, liefde en geduld.

Curriculum Vitae

Francisca Velkers werd op 12 december 1975 geboren te Rotterdam. Ze groeide op in Rotterdam-Zuid en behaalde haar Atheneum diploma aan CSG Johannes Calvijn te Rotterdam in 1994. Daarna heeft ze Biologie gestudeerd aan de Rijksuniversiteit Leiden en behaalde ze het propedeusediploma in 1995. In 1996 werd ze ingeloot voor de studie Diergeneeskunde. Na het behalen van het doctoraaldiploma in 2000 heeft ze gedurende negen maanden gewerkt bij de afdeling Biomedische Natuurkunde en Technologie aan het ErasmusMC te Rotterdam, waar ze onderzoek deed naar bekkenbodemspieren bij mensen. Daarna hervatte ze haar studie Diergeneeskunde met het volgen van de co-schappen. Tijdens het laatste jaar werd het mono-traject Pluimvee gevolgd dat in 2003 werd afgesloten met het behalen van het dierenartsexamen. Na een aantal maanden te hebben gewerkt als dierenarts tijdens de vogelpestuitbraak in Nederland werd ze aangesteld bij het departement Gezondheidszorg Landbouwhuisdieren van de Faculteit Diergeneeskunde aan de Universiteit Utrecht. Bij de afdeling Pluimveegezondheidszorg was ze werkzaam als junior docent/specialist in opleiding. Daarnaast was ze voor haar werk als promovendus (AIO) aangesteld bij de afdeling Kwantitatieve Veterinaire Epidemiologie van Wageningen UR (University & Research centre) en bij Epidemiologie van Infectieziekten van het departement Gezondheidszorg Landbouwhuisdieren. Het uitgevoerde onderzoek is beschreven in dit proefschrift. De Master of Science opleiding Veterinary Epidemiology and Economics werd in deeltijd gevolgd vanaf 2003 en werd afgerond in 2006. Inmiddels heeft Francisca een vaste aanstelling bij het departement Gezondheidszorg Landbouwhuisdieren als docent en onderzoeker.

List of Publications

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Velkers, F.C., Bouma, A., Stegeman, J.A., de Jong, M.C.M., 2009. Transmission of *Eimeria acervulina* in a group of broiler chickens. Proceedings of the 12th Symposium of the International Society for Veterinary Epidemiology and Economics (ISVEE XII), 10-14 August 2009, Durban, South Africa, p. 578.

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Training and Supervision Plan

Training and Supervision Plan
Wageningen Institute of Animal Sciences (WIAS)

Education and Training	Year
The Basic Package (3 credits)	
WIAS Introduction Course	2005
WIAS Course Philosophy of Science and Ethics	2005
Scientific Exposure (11 credits)	
International conferences	
15th World Veterinary Poultry Association Congress, Beijng, 10-15 Sept.	2007
IXth International Coccidiosis Conference, Foz do Iguassu, 19-23 Sept.	2005
Seminars and workshops	
European Coccidiosis Discussion Group, Compton, 21 May	2009
Annual Meeting Dutch Society for Veterinary Epidemiology and Economics, 12 Febr. 2004, 16 Febr. 2006, 15 Febr. 2007	2004 + 2006 2007
WIAS Science Day, Wageningen, 17 Febr. 2005, 9 March 2006	2005 + 2006
Workshop Epidemiological Modelling by Gary Smith, Utrecht, 15 Sept.	2004
Poultry Coordination Centre Meeting "Epidemiology", Utrecht, 7 Okt.	2003
Presentations	
Transmission of *Eimeria acervulina* in a group of broiler chickens, 12th Symposium of the International Society for Veterinary Epidemiology and Economics, Durban, 10-14 Aug., poster	2009
Transmission of *Eimeria acervulina* in broiler chickens, 15th World Veterinary Poultry Association Congress, Beijng, 12 Sept., oral	2007
Transmission of *Eimeria acervulina* in broiler chickens, Annual Meeting Dutch Society for Veterinary Epidemiology and Economics, Utrecht, 15 Febr., oral	2007
De ontwikkeling van een studie naar transmissie van coccidiose in vleeskuikens, Groep Groot Dagen, Arnhem, 22 Nov., oral	2006
Quantification of transmission of *Eimeria acervulina* in broilers: a one-to one transmission experiment, 11th Symposium of the International Society for Veterinary Epidemiology and Economics, Cairns, 6-11 Aug., poster	2006
Quantification of transmission of *Eimeria acervulina* in broilers: a one-to one transmission experiment, WIAS Science Day, Wageningen, 9 March, oral	2006
Comparison of *E. acervulina* oocyst counts in single droppings of broilers and in droppings collected during 24 hours, IXth International Coccidiosis Conference, Foz do Iguassu, 23 Sept., oral	2005
In-Depth Studies (21 credits)	
Disciplinary and interdisciplinary courses	
Poultry Pathology Course, Veterinary Extension Services	2009
Molecular Diagnostics IV, ErasmusMC	2009
Poultry Animal Health and Production Course, GD-Animal Health Service	2005

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Education and Training	Year
MSc level courses	
Msc Veterinary Epidemiology and Economics: "Clinical Epidemiology", "Veterinary Epidemiology", "Modelling of Infectious Diseases", "Risk Analysis", "Bayesian analysis"	2006
Msc Veterinary Epidemiology and Economics: "Modern Statistical Methods"	2004
Professional Skills Support Courses (3 credits)	
Applying for Grants, NWO Talent Class	2010
Negotiating, Networking, NWO Talent Class	2009
Project and Time Management, Wageningen Graduate Schools (WGC)	2007
Interpersonal Effectiveness, Team Time Trainingen	2007
Techniques for Writing and Presenting a Scientific Paper, WGC	2004
Didactic Skills Training (21 credits)	
(including preparing of course materials)	
Lecturing *(all poultry related)*	
Viral diseases, Medication, Diseases in Backyard Poultry	2003-2010
Vaccination Methods and Schemes, Diagnostic Techniques	2003-2010
Supervising practicals and excursions	
Parasitological Examination Practical, Poultry Farm Visits, Supervising SWOT Analyses Poultry Farms: veterinary medicine clinical rotation students	2003-2010
Clinical Lessons/Post-mortem Examination: graduate veterinary medicine students	2005-2010
Elective Course Clinical Pathophysiology: graduate veterinary medicine students	2006
Supervising theses	
Msc Animal Science (minor thesis): Huiming Liu	2010
Msc Veterinary Medicine: Robin Zijlmans	2010
Msc Veterinary Medicine: Chantal Schoenmaker	2009
Msc Veterinary Medicine: Christiaan ter Veen	2008
Msc Veterinary Epidemiology and Economics: Tariq Halasa	2004-2005
Management Skills Training (1 credit)	
Organisation of seminars and courses	
Parasitological Examination of Poultry Faeces: course for poultry lecturers of the Faculty of Veterinary Medicine, Utrecht University	2005
Membership of boards and committees	
Interim Member Research Committee Department of Farm Animal Health, Utrecht University	2005-2006
Education and Training Total	**60**
* one ECTS credit equals a study load of approximately 28 hours	

Colophon