SUSCEPTIBILITY OF BROILERS TO COLIBACILLOSIS

Opportunities of Challenge Testing and Indicator Traits in Selection Strategies

GEVOELIGHEID VAN VLEESKUIKENS VOOR COLIBACILLOSIS
Potenties van challengetesten en indicator kenmerken in selectiestrategieën
(met een samenvatting in het Nederlands)

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ABSTRACT: This dissertation aimed to evaluate broiler susceptibility to colibacillosis and the potential of genetic selection to reduce broiler susceptibility to colibacillosis. A challenge experiment with *E. coli* at 7 days of age was carried out on eight broiler genotypes: five pure broiler lines, a slow-growing line, and two 2-way crosses of the pure-lines. Based on the results from this experiment, a sensible definition of the susceptibility to colibacillosis was defined, and the indication of genetic variation in the susceptibility was found, suggesting that selection for reduced susceptibility is possible. Maternal antibodies did not have an effect on susceptibility, but there were indications of genetic variation in the changes in thyroid hormones in response to challenge as well as in the antibody response to challenge. Difficulties in evaluating immunological variables hinder attempts to improve animal health through selection on immunological variables. A model was developed that describes immunocompetence development as well as kinetics of immunoresponsiveness to a pathogenic challenge in an individual chick. This model provides a useful tool in the definition of appropriate challenge and measurement strategies when evaluating immunocompetence and immunoresponsiveness. The model was expanded into a stochastic model that describes a population of individual chicks with variation among them as well as stochastic variation within individuals across age. The model predicts that heteroscedasticity in variance across age decreases with increasing challenge age, and that minimum probability to detect a given difference in immunocompetence or responsiveness at another age than the selection age increases with increasing selection age. Therefore, a high challenge age, at which maternal immunity no longer has influence, is preferable. Selection against susceptibility to colibacillosis should aim at reducing the incidence of colibacillosis at commercial broiler level and be based on a combination of information on indicator and clinical traits. Because the economic importance of bacterial diseases as a whole is much higher than that of colibacillosis alone, changing the breeding goal to reducing the incidence of bacterial diseases as a whole is sensible.

Keywords: *Broiler, Colibacillosis, Susceptibility, Selection, Immunocompetence, Immunoresponsiveness, Challenge, Indicator traits*

Contents

Chapter 1: General Introduction

1.1 Bacterial Infections in the Broiler Industry

Diseases caused by bacteria in the broiler industry are a main concern in broiler industry, and considerable research emphasis is placed on limiting the impact of these diseases through development of new vaccines and alternative disease control strategies (NCRA, 2004; Laughlin, 2005). Bacterial infections are an important welfare issue for the chickens, but they are also economically important, because diseases are a significant financial cost to the broiler industry, mainly due to mortality, growth retardation, uniformity reduction and condemnations (Goren, 1991; Vandemaele et al., 2002; McKissick, 2006). The main cause of broiler mortality and the economically most significant group of infectious poultry diseases is respiratory diseases, including both primary and secondary bacterial diseases. Bacterial diseases of major economic importance include cellulitis (caused by *Escherichia coli* (*E. coli*) (Messier et al., 1993; Peighambari et al., 1995)), colibacillosis (Vandekerchove et al., 2004; Laughlin, 2005), salmonellosis, and fowl cholera (caused by *Pasteurella multocida*) (Bumstead, 2003). In fact, probably 70-90% of all abbatoir condemnations are accounted for by secondary bacterial infections (systemic disease and airsacculitis) and cellulitis (Laughlin, 2005; NCRA, 2004).

The ban of the use of all prophylactic antibiotic growth promoters (AGP's) enforced by the EU from the 1st of January 2006 (Gussem, 2004), has further stimulated the demand for alternative disease control strategies against bacterial infections (Feighner and Dashkevicz, 1987; Zekarias et al., 2002). Experience from Sweden (banned all AGP's in 1986) and Denmark (banned avoparcin and virginiamycin in respectively 1995 and 1998) has shown that, in some cases, the removal of AGP's resulted in a decline in animal health, e.g. increased mortality and dioarrhea (Casewell et al., 2003). This decline has been counteracted by a temporary increased use of therapeutic antibiotics and alternative growth promoters, such as ionophores (Tornøe, 2003; Wierup, 2003). Subsequently, no negative effects of the AGP ban have been observed on the health in neither Sweden nor Denmark. In the Dutch broiler industry, the therapeutic use of antibiotics has increased annually from 1990 till 2004, and of the used antibiotics, the highest contribution is by quinolones, which is attributed to mainly *E. coli* infections. The reason for the increased therapeutic use of antibiotics is unknown (MARAN, 2002; MARAN, 2004), but the ban of various AGP's may be a part of the reason.

The threat of foodborn diseases in humans, caused by mainly *Campylobactor* and *Salmonella*, but also by *E. coli* (Mead and Griffin, 1998; Jorgensen et al., 2002; Jeffrey et al., 2004; CDC, 2005), further stresses the importance of limiting bacterial infections

in the broilers. Recently, the occurrence of septicaemia, airsacculitis, and cellulitis in broiler flocks has been shown to be positively associated with carcass microbial loads (*E. coli*, *Campylobactor*, and *Salmonella*). This demonstrates that broiler health is not only important for economic and welfare concerns, but also for human food-safety concerns (Dawe, 2004). Foodborne diseases have increased significantly during the last two decades (Rocourt et al., 2003), and world wide, foodborne diseases, along with waterborne diseases, are responsible for approximately 1.8 million deaths per year (WHO, 2006). Of the three pathogens that most frequently cause foodborne diseases because of contaminated broiler meat, C*ampylobactor* and *Salmonella* are mostly known as zoonoses in broilers, whereas *E. coli* is more frequently associated with a decline in broiler health. Because *E. coli* thus is the pathogen with highest importance for broiler health and human food-safety combined, this dissertation will focus on *E. coli*.

1.2 The *E. coli* Bacterium

E. coli is a gram-negative, flagellated bacterium, which is a part of the normal flora in chickens, both in the digestive- and the upper respiratory tracts. It is in general of low virulence for chickens (Hofstad et al., 1978; Nakamura et al., 1992). Pathogenic *E. coli* is limited to a small range of serotypes, which include O78K80, O1:K1, and O2:K1 (Hofstad et al., 1978; Wray et al., 1996; Mead and Griffin, 1998). Virulence factors of avian pathogenic *E. coli* include pilus adherence, serum resistance (among others indicated by the capsular polysaccharide K1), type 1 and P fimbriae (especially F11 fimbriae, which protects against phagocytosis), curli fimbriae, flagella (motility ability), and assimilation of iron mediated by aerobactin production (Vidotto et al., 1990; Pourbakhsh et al., 1997; Vidotto et al., 1997; Dho-Moulin and Fairbrother, 1999; Ginns et al., 2000; La Ragione et al., 2000; Edelman et al., 2003; Mellata et al., 2003; Vandekerchove et al., 2004).

1.2.1 Colibacillosis

Several important diseases in broilers are associated with *E. coli* as a primary causal, or secondary aggravation, factor. Omphalitis, coligranuloma, osteomyelitis, synovitis, salpingitis, airsacculitis, cellulitis, pericarditis, perihepatitis, swollen head syndrome, panophthalmitis, and colisepticaemia are all results of, or can be associated with, infection with *E. coli* (Hofstad et al., 1978; Wray et al., 1996). The term respiratory colibacillosis, or simply colibacillosis, is used in association with air sac disease, pericarditis, perihepatitis, and colisepticaemia, and is, as the term suggests, an infection with *E. coli* that has entered the broiler via the respiratory route. Mortality usually stays

below 5% but the proportion of disease cases often reaches more than 50% (Wray et al., 1996; Vandekerchove et al., 2004).

In broilers, the prevalence of colibacillosis is lower in the first half of the production period than in the second half (Goren, 1978). In the first half, colibacillosis occurs as a primary infection, and in the second half, it predominantly occurs as a secondary infection. Potential primers range from environmental factors, i.e. dust and ammonia levels in the stable, to viruses, such as infectious bronchitis virus, Newcastle disease virus, and infectious bursal disease virus, and vaccinations against these viruses (Hofstad et al., 1978; Cook and Mockett, 1995; Matthijs et al., 2003; Jeffrey et al., 2004). The higher prevalence in the second half of the production period has been suggested to be associated with an exponential increase of the bacterial flora in the barn, the fading of maternal antibodies, and the physiological strain on the broilers caused by their high growth rate (Carlson and Whenham, 1968; Goren, 1981). Colibacillois is, however, not uncommon in the first half of the production period either, which is probably partly due to vertical transmission of pathogenic *E. coli* (Hofstad et al., 1978; Wray et al., 1996).

Traditional management strategies such as vaccinations and preventive use of, or therapeutic treatment with, antibiotics are insufficient in preventing colibacillosis. Vaccination against *E. coli* is limited to homologous protection, and vaccination against primers to secondary colibacillosis may function as a primer itself. Use of antibiotics is expensive and treatment often does not result in sufficient recovery before slaughter (Goren, 1991; Vandemaele, 2002a and b). In addition, preventive use of antibiotics has resulted in increased antibiotic resistance of *E. coli* (resistant to at least one of seven antibiotics that have been frequently used in poultry on veterinary prescription). The prevalence of resistant *E. coli* in faecal samples from broilers has been shown to be up to 82%, and up to 26% of these resistant samples were shown to be highly resistant (antibiotics resulting in a maximum inhibition of bacterial colony growth of 50%) (Van den Bogaard et al., 2001). Increasing hygienic standards is also not sufficient in prevention of colibacillosis, because exposure of broilers to bacteria such as *E. coli* is unavoidable under practical circumstances. Even if the risk of infection with *E. coli* is reduced by an increase in the current hygienic standards, the susceptibility of broilers to colibacillosis may be increased, because the immune system functionality is dependent on among others environmental stimuli (Henryon et al., 2003).

It can be concluded that there is a demand for alternative or supplementive control strategies for colibacillosis, as was the case for bacterial infections in general. Genetic selection may offer such an eligible supplementive to the traditional management strategies (Adams and Templeton, 1998; Detilleux, 2001; Stear et al., 2001).

1.3 Genetic Selection against Susceptibility to Colibacillosis

Genetic selection to reduce susceptibility to colibacillosis requires the presence of genetic variation between chicks in susceptibility. There are indications that such genetic variation exists. For example, genetic differences in susceptibility to colibacillosis have been observed in various line-comparison experiments. These experiments include divergently selected experimental lines for antibody response to *E. coli* vaccination (Leitner et al., 1992; Yonash et al., 2000; Yunis et al., 2000; Yunis et al., 2002), experimental lines selected for antibody response to sheep erythrocytes (Gross, 1990; Dunnington et al., 1991), or selection lines for high and low juvenile body weight (Reddy et al., 1975; Praharaj et al., 1996). Other experiments that demonstrated differences between breeds included local breeds and commercial broilers or pure-lines (Bumstead et al., 1989; Praharaj et al., 1999; Rama Rao et al., 1999; Praharaj et al., 2002; Reddy et al., 2002). Observed differences between different lines are as high as 67% in the occurrence of lesions, and range from 3 to 87% in mortality (Smith et al., 1985; Bumstead et al., 1989; Gross, 1990; Praharaj et al., 1996; Rama Rao et al., 1999; Yunis et al., 2002). Challenge protocols have differed considerably between experiments though. This complicates evaluation of the possible presence of a genetic component in the susceptibility to colibacillosis, because the definition of the susceptibility to colibacillosis differs (if defined at all). Observed responses to a given challenge may reflect susceptibility to *naturally* occurring colibacillosis differently depending on the challenge protocols. Protocols differed regarding challenge route (aerosolly, intranasally, intravenously, and via air sacs), age at challenge (from 8 to 43 days of age) and infection type (*E. coli* vaccination, primary *E. coli* challenge, and secondary *E. coli* challenge in combination with infectious bronchitis virus, New castle disease virus, infectious bursal disease virus, or *Mycoplasma gallisepticum*). Evaluation criteria have also differed considerably from only recording mortality to recording pericarditis and/or airsacculitis or antibody response.

Heritabilities of susceptibility to colibacillosis as such, i.e. the amount of additive genetic variation relative to phenotypic variation in a population, have not been estimated. However, heritability estimates of antibody response to *E. coli* infection range from 0.21 to 0.72 (Pitcovski et al., 1987; Yonash et al., 1996).

The heritability estimates demonstrates that genetic variation within lines is present but the nature of this variation is largely unknown. The variation is probably at least partly related to the major histocompatibility complex (MHC) though, because the MHC has been associated with immune response to *E. coli*, and in some cases resistance to *E. coli*. Macklin et al. (2000, 2002) found a relationship between chicken MHC haplotypes and the susceptibility to cellulitis, where the haplotype B^{21} was identified as having a

higher probability of initial infection. An influence of the MHC on the antibody response to *E. coli* vaccination at 10 days of age in broiler-type chickens has also been indicated (Uni et al., 1993). However, no positive association between the antibody response and resistance to challenge with *E. coli* could be found (Yonash et al., 1996). In lines differing in resistance to *E. coli* (Heller et al., 1992; Leitner et al., 1992), the MHC was found to be associated with variation in immune response (Yonash et al., 1999). Further, a QTL, which is not linked to the MHC, has been significantly associated with both antibody response and resistance to *E. coli* in broilers (Yonash et al., 2001).

In summary, susceptibility to colibacillosis may have a genetic component. However, before implementation of selection against susceptibility to colibacillosis may be realized, the best procedure to evaluate the susceptibility must be identified and the amount of genetic variation present in commercial pure-lines must be determined.

1.4 Outline and Main Purpose of Dissertation

The main objectives of this dissertation were to evaluate broiler susceptibility to colibacillosis and the potential of genetic selection to reduce broiler susceptibility to colibacillosis.

In Chapter 2, broiler susceptibility to colibacillosis is determined based on two (repeated) challenge experiments, using a primary *E. coli* challenge, and the relationship between the susceptibility and growth retardation is investigated. This chapter provides important information for the design of effective selection strategies to reduce broiler susceptibility. In Chapter 3, the presence of genetic variation in susceptibility to colibacillosis is investigated based on differences between genetic lines in the experiments described in Chapter 2. Eight different genetic lines, including three dam-lines, two sire-lines, two pure-line crosses, and a slow-growing genotype, were investigated. In Chapter 4, the association of susceptibility to colibacillosis with maternal antibodies, antibody response and thyroid hormones is investigated along with the presence of genetic variation (line differences). The association of susceptibility to colibacillosis and thyroid hormones was expected provide increased insight into the biological background of growth retardation as a response to *E. coli* infection, because of their key role in metabolism.

A large number of indicator traits reflecting susceptibility to infections have been suggested. In this dissertation, the potential of immunocompetence, i.e. immunological ability to resist and recover from infection, as an indicator trait is investigated by computational modelling. In Chapter 5, a deterministic simulation model that describes immunocompetence development as well as kinetics of immunoresponsiveness to a pathogenic challenge in an individual chick is presented. The model is fitted to published

data and its utility for the definition of appropriate challenge and measurement strategies when evaluating immunocompetence and immunoresponsiveness is illustrated. In Chapter 6, a stochastic model was developed by expanding the model described in Chapter 5 to a population of individual chicks. The characteristics of the variation in the model are compared to observed variation in literature, and the consequences of this variation for statistical power of genotype comparisons and selection are illustrated. In Chapter 7.1, the experimental design used for the experiment described in Chapters 2, 3, and 4 is evaluated in the context of the results, newly acquired knowledge, and the issues of experimental design that were explored with the immune system model described in Chapter 5. In Chapter 7.2, the breeding goal for selection for reduced susceptibility to colibacillosis is considered. Potential indicator traits and clinical traits are evaluated as traits for selection in Chapter 7.3, and in Chapter 7.4, the information sources on which data should be collected and the level at which data should be collected for genetic evaluations is discussed. In Chpater 7.5, some additional considerations for a breeding program including selection against susceptibility to colibacillosis are discussed. Finally, in Chapter 7.6, the implementation of selection against susceptibility to colibacillosis in practice is discussed.

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Chapter 2: Defining Susceptibility of Broiler Chicks to Colibacillosis

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Abstract: This study aimed to define the susceptibility of broilers to colibacillosis through quantification of clinical responses and to examine the relationship between susceptibility and growth retardation. A challenge experiment was carried out twice. In each trial, 192 chicks were challenged intratracheally with *E. coli* at 7 days of age and 160 chicks served as controls. Surviving chicks were euthanized at 14 or 15 days. Parameters measured were: daily mortality, lesion scores, body weight at 1, 4, 7, 10, 12 and 14 or 15 days and feeding behaviour at 6, 11 and 13 days. The results were reproducible, and increasing susceptibility to colibacillosis was defined by four categories: chicks without lesions, chicks with airsacculitis but no systemic lesions, chicks with systemic lesions, and chicks that die. Increasing susceptibility was associated with increasing growth retardation, but growth retardation was not inevitably linked to challenge with *E. coli*.

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2.1 Introduction

Colibacillosis, which is caused by *E. coli*, causes considerable economic and welfare problems in broilers (Goren, 1991; Bettelheim, 1994; Vandemaele et al., 2002b), due to its frequent occurrence and its adverse effects on growth and health. Clinical disease consists of respiratory signs, growth retardation, reduced feed intake and increased mortality (Goren, 1991; Vandemaele et al., 2002b). Airsacculitis and fibrinous polyserositis (e.g. pericarditis, perihepatitis and peritonitis) are the main gross lesions, and septicaemia, sometimes acute, is also common (Whiteman et al., 1989).

Good hygiene, vaccination and therapeutic treatment do not provide sufficient protection against colibacillosis. Vaccination against *E. coli* is limited to homologous protection, and the vaccines used against the primary agents that provoke secondary colibacillosis may themselves act as primary agents. Therapeutic treatment is expensive, often does not result in sufficient recovery before slaughter and causes increased resistance of *E. coli* to antibiotics (Vandemaele et al., 2002a; Vandekerchove et al.,

2004). Breeding for reduced susceptibility to colibacillosis may contribute to its prevention, but in order to do so a meaningful breeding goal and traits to measure must be defined. In other words, susceptibility to colibacillosis must be clearly defined in order to distinguish between more or less susceptible individuals. Previous studies do not provide sufficient information on the clinical responses, or the associations between responses, to provide a rational definition of susceptibility to colibacillosis, and assumptions have thus been made. A thorough investigation of responses to infection with *E. coli* was therefore considered necessary.

Previously, different assumptions have been made on the relative severity of lesions. Some studies have suggested a relationship between severity and the type of lesions, with systemic infections (pericarditis) being counted more severe than airsacculitis (Praharaj et al., 1996), and thus implying that chicks with systemic lesions are more susceptible than those with airsacculitis.

This was based on the colibacillosis pathogenesis (i.e. systemic infection developing after initial infection of the respiratory system), and linked susceptibility with the infection stage. However, this does not necessarily directly reflect differences in susceptibility. Other studies have assumed that the relative severity of the lesions is not related to type of lesion and have reported lesion scores as population means based on the total scores of individuals (the sum of the lesion scores of all lesion types) (Peighambari et al., 2000; Matthijs et al., 2003).

Increased knowledge on the relative severity of lesions is necessary to establish a meaningful definition of susceptibility to colibacillosis, and the association between lesions and growth retardation and feed intake. Previous studies have shown that, on average, *E. coli* infected chicks show growth retardation and that the amount of retardation is related to dose of infection (Dunnington et al., 1991; Maatman et al., 1993; Matthijs et al., 2003). However, these studies did not provide information on the association between growth retardation and lesion type or severity, which would facilitate a meaningful definition of susceptibility to colibacillosis. To the authors' knowledge, there is also no information on the association between feed intake and mortality, lesion type or severity, which would also help to establish a meaningful definition of susceptibility to colibacillosis.

The aim of this study was therefore to define the susceptibility to colibacillosis in terms of mortality, lesions (airsacculitis, pericarditis and perihepatitis), growth and feeding behaviour (to provide information on feed intake) (Pym & Nichols, 1979; Nir et al., 1994). Colibacillosis was defined as clinical disease, and not merely infection with *E. coli*.

2.2 Materials and Methods

A challenge experiment was carried out on a population consisting of multiple broiler lines and crosses to ensure a broad genetic inference in the results. The experiment was performed twice in order to evaluate the reproducibility of the results. The results on the individual lines and crosses are the subject of another paper due to the large amount of data generated (Ask et al., 2006).

2.2.1 Chicks

Eggs were incubated and hatched for the two trials at the Spelderholt Poultry Research Centre in Beekbergen, The Netherlands. The eggs originated from six broiler lines and two crosses: two sire (A2, E3), three dam (A3, E4, E5), one slow-growing line (3), a sire cross (A2× E3) and a dam cross (A3× E4). In the text, the lines and crosses are referred to as genotypes.

2.2.2 Experimental Design

At 1 day of age (day of hatch), the chicks were individually tagged and divided into a challenge and control group, each in four pens. In each trial, there were 192 chicks in the challenge group (48 chicks per pen), and 160 chicks in the control group (40 chicks per pen). Greater numbers were placed in the challenge group to anticipate losses due to mortality. Genotype and gender were equally represented: in each pen in the challenge group, there were three males and three females per genotype. In the control group, there were three males and two females per genotype in each of two of the four pens, and two males and three females per genotype in the other two pens.

At 7 days of age, all chicks in the challenge group were challenged (see later), and the experiment was terminated at approximately 2 weeks of age. The surviving chicks were stunned by electrocution and euthanized by bleeding: one-half of the birds in the challenge group and one-half of the control group were euthanized at 14 days of age, and the others at 15 days of age.

2.2.3 Housing

The challenge and control groups were kept on litter in separate, but identical, climate-controlled chambers to avoid horizontal infection of control chicks. Each pen measured 1.54× 1.75 m^2, with walls 0.5 m high. Feed and water was provided ad libitum. A daily schedule of 20:4 h light:dark was used, commencing with lights on at 06:00 h. Environmental temperature followed a standard schedule, starting at 348C at 1 day of age followed by a gradual decline to 248C at 15 days of age. The relative humidity was kept at 50%.

2.2.3 Challenge

All chicks in the challenge group were inoculated intratracheally with 0.3 ml of 1:100 phosphate-buffered saline solution of *E. coli* strain 506 (serotype O78K80) cultured in glucose peptone broth. *E. coli* 506 was a flumequine-resistant strain isolated from the inflamed pericardium of a commercial broiler suffering from natural colibacillosis (van Eck and Goren, 1991). The inoculation was carried out using a 1.0 ml syringe fitted with a blunt-ended pipette tip (catalogue number 4862; Corning, New York, USA) and the doses per bird were $10^{6.0}$ colony-forming units in trial 1 and $10^{5.8}$ colony-forming units in trial 2. In the first trial, 25 chicks from one pen were each given 0.5 ml *E. coli* inoculum (corresponding to $10^{6.3}$ colony-forming units), but four chicks showed signs of suffocation within 15 min. The volume was therefore adjusted to 0.3 ml for the remainder of the chicks and those that had received 0.5 ml were omitted from the analyses. All chicks in the control group were inoculated intratracheally with 0.3 ml phosphate-buffered saline.

2.2.4. Recording of Traits

The body weight of individual chicks was recorded at 1, 4, 7, 10 and 12 days of age. In addition, chicks in one half of the control and challenge pens were weighed at 14 days of age and the other half at 15 days of age. Mortality was recorded each morning.

Feeding behaviour was recorded by a single observer at 6, 11 and 13 days of age in both the control and challenge groups. Each pen was observed once in the morning (between 08:00 and 12:00 h) and once in the afternoon (between 13:00 and 17:00 h): the control pens were always observed first, and the challenge pens always last. An observation period lasted 20 min per pen and, at intervals of 40 to 60 sec, the identities of chicks that were eating (pecking at feed) were recorded. Individual identification was enabled through dorsal crayon stripe(s) of different colours. A different colour was used for each genotype, and to distinguish genotypes within a pen one to three stripes were applied; either from neck to tail (males) or from wing to wing (females).

Observations made at 6 days of age in trial 1 were omitted from the analysis due to recording problems.

At postmortem examination gross lesions of the right and left thoracic air sac (airsacculitis), the heart (pericarditis) and the liver (perihepatitis) were scored macroscopically. Thoracic air sac lesions were considered to represent *E. coli* pathology of the respiratory tract, and pericarditis and perihepatitis were considered to represent systemic *E. coli* pathology. Lesion scoring was carried out blind and performed as described by van Eck and Goren (1991) using the following scale: 0 = no lesions, 0.5 =

one yellow or brown pinhead-sized spot indicative of inflammation, 1 = two or more pinhead-sized spots indicative of inflammation, 2 = thin layer of fibrinous exudate on various locations, and 3 = thick and extensive layer of fibrinous exudate.

Chicks that died during the experiment were dissected and examined macroscopically but lesions were not scored. Bacteriological examination was performed on the spleens of all chicks that died during the experiment, and the antibiotic sensitivity of the *E. coli* isolates was compared with that of *E. coli* strain 506 as described by Velkers et al. (2005). The cause of death was considered to be colibacillosis if there were signs of airsacculitis, pericarditis or perihepatitis or if *E. coli* 506 could be isolated from the spleen.

2.2.5 Data analysis

Mortality and lesion traits were: incidence of mortality (in the challenge group, post-inoculation, as the percentage of total number of chicks at 7 days of age), lesions in the right and left thoracic air sac (RA and LA), pericarditis, perihepatitis, airsacculitis (the sum of RA and LA) and systemic lesions (the sum of pericarditis and perihepatitis). The feeding behaviour was defined as feeding or not feeding during each observation period, and the prevalence of chicks that did not show feeding behaviour was used as a trait. Observations of feeding behaviour on chicks that died during the experiment were also included in the analyses.

The Wilcoxon (or Kruskal-Wallis) test was used for comparisons of the incidence of mortality, the prevalence of lesions and the proportion of chicks not feeding. When sample sizes were not sufficiently large, Pearson's chi-square test was used for the comparisons.

Body weights at 1, 4, 7, 10 and 12 days of age were abbreviated as BW1, BW4, BW7, BW10 and BW12, respectively. The body weights at 14 or 15 days of age were treated as one trait, abbreviated as BW14. Body weights of chicks that died during the experiment were also included in the analyses. Body weights were adjusted by means of an analysis of variance, and the *F* test was applied to test for differences in body weights between trials. The model was:

$$Y_{ijklm} = \mu_{ijklm} + \text{TRIAL}_i + \text{TREATMENT}_j + \text{SEX}_k + \text{GENOTYPE}_l + \text{DAY1415}_m \text{ (for BW14)} + \varepsilon_{ijklm},$$

where Y_{ijklm} is the body weight in the *i*th trial, the *j*th treatment (challenge or control group), the *k*th sex, the *l*th genotype and the *m*th DAY1415, DAY1415 is the age on which the final measurement of body weight was taken, and ε_{ijklm} is the random residual effect.

The *F* test was also applied to test for differences in body weights between the challenge and control groups per trial, using the following model:

$$Y_{ijkl} = \mu_{ijkl} + \text{TREATMENT}_i + \text{SEX}_j + \text{GENOTYPE}_k + \text{DAY1415}_l\,(\text{for BW14}) + \varepsilon_{ijkl}$$

where Y_{ijkl} is the body weight in the *i*th treatment (challenge or control group), the *j*th sex, the *k*th genotype and the *l*th DAY1415, and ε_{eijkl} is the random residual effect.

The *t* test was applied to test for differences between the control group and the following groups within the challenge group per trial: chicks without lesions, chicks with airsacculitis but no systemic lesions, chicks with systemic lesions, and chicks that died during the experiment. Tukey's adjustment (the Tukey-Kramer method) was used to correct for multiple comparisons. The model was:

$$Y_{ijkl} = \mu_{ijkl} + \text{LESION}_i + \text{SEX}_j + \text{GENOTYPE}_k + \text{DAY1415}_l\,(\text{for BW14}) + \varepsilon_{ijkl}$$

where Y_{ijkl} is the body weight in the *i*th lesion (control group and the following groups within the challenge group: chicks without lesions, chicks with airsacculitis but no systemic lesions, chicks with systemic lesions, and chicks that died during the experiment), the *j*th sex, the *k*th genotype and the *l*th DAY1415, and ε_{ijkl} is the random residual effect.

Growth retardation was defined as:

$$\frac{\overline{BWage}_{cont} - \overline{BWage}_{chal}}{\overline{BWage}_{cont}} \times 100$$

where $\overline{BWage}_{cont}$ and $\overline{BWage}_{chal}$ are the least-square means of the body weights at a certain age in the control and challenge group, respectively. The *t* test was applied to test for differences in growth retardation, using Tukey's adjustment to correct for multiple comparisons.

2.2.6 Ethics

The experiment was approved by the Animal Ethics Committee (Dierexperimentencommissie, Utrecht University, The Netherlands), and chicks were handled accordingly. The Animal Ethics Committee based its decision on ''De Wet op Dierproeven'' (1996) and on the ''Dierproevenbesluit'' (1985) (http://www.nca-nl.org/).

2.3 Results

2.3.1 Mortality

Before the inoculation (1 to 7 days of age), eight chicks in the control group and three in the challenge group died, all for unknown reasons. In the control group, one chick died post inoculation, also for unknown reasons. Overall mortality in the challenge group amounted to 45 chicks in trial 1 and 62 in trial 2. Typical macroscopic *E. coli* pathology was present in all but 10 chicks in trial 1 that had died 1 day post inoculation, and it was present in all but six chicks in trial 2, of which five had died 1 day post inoculation and one died 6 days post inoculation. *E. coli* isolates that matched the sensitivity pattern of strain 506 were recovered from the spleens of all chicks that died during the experiment, except that from the chick in trial 2, which died 6 days post inoculation and did not show macroscopic *E. coli* pathology.

The total mortality incidence per trial is presented in Table 2.1 and there was no significant difference between trials 1 and 2 ($P = 0.586$). The mortality per day in each trial is given in Figure 2.1. The mortality in trial 1 showed a clear peak at 8 days of age (1 day post inoculation), and there was also a second, but less pronounced, peak between 11 and 13 days of age (4 to 6 days post inoculation). The mortality in trial 2 showed a clear peak at 13 days of age (6 days post inoculation) only. Significant differences in mortality pattern between trials were thereby apparent: at 1 day post inoculation (5.94% more mortality in trial 1 than 2, $P < 0.001$) and at 6 days post inoculation (5.35% less mortality in trial 1 than 2, $P < 0.001$).

Table 2.1. *Incidence of mortality, lesion prevalence (%) and scores of broiler chicks challenged with E. coli in two trials*

				P(row)[b]	
	Trial 1	Trial 2	*P*(trial)[a]	Trial 1	Trial 2
Mortality	27.3	32.5	0.287	0.835	0.319
Lesions[c], total:	25.8	28.1	0.613		
Airsacculitis (lesions in right or left thoracic air sac)	25.8	26.0	0.950	0.114	0.280
Systemic lesions (pericarditis or perihepatitis)	18.6	21.4	0.510		
RA (lesions in right thoracic air sac)	23.4	25.5	0.634	0.598	0.084
LA (lesions in left thoracic air sac)	21.0	18.2	0.515		
Pericarditis	18.6	20.8	0.590	0.667	0.109
Perihepatitis	16.8	14.6	0.570		
Airsacculitis but no systemic lesions and at least one score of 3[d]	0.0	15.4	< 0.001	< 0.001	< 0.001
Systemic lesions and at least one score of 3[d]	93.6	87.8	0.415		

[a] *P*(trial) is the p-value of the Wilcoxon test for differences between trial 1 and 2.

[b] *P*(row) is the p-value of the Wilcoxon test for differences between pairs of rows.

[c] Lesions were not scored for chicks that died during the experiment.

[d] In percent of chicks that had at least one lesion score > 0.

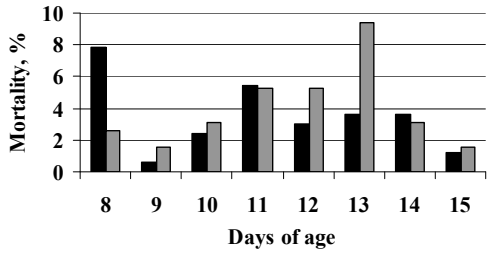

Figure 2.1. *Daily mortality of broiler chicks challenged with E. coli as a percentage of the total number of chicks at 7 days of age in trial 1 (black columns) and trial 2 (grey columns).*

2.3.2 Lesion traits

The prevalence of lesions in the challenge group per trial is presented in Table 2.1. No significant differences between trials were found in the prevalences of lesions. On average, 27% of the chicks developed colibacillosis expressed as lesions. No significant differences were found in the prevalence of airsacculitis or systemic lesions. There was a tendency to a higher prevalence of RA than LA in trial 2 (7%, $P = 0.084$), but not in trial 1, and no significant differences were found in the prevalence of pericarditis or perihepatitis. In both trials, the prevalence of systemic lesions, which was accompanied by at least one lesion (RA, LA, pericarditis or perihepatitis) of the highest severity (3), was significantly higher than the prevalence of airsacculitis accompanied by at least one lesion with the highest severity.

The relationship between the severity of RA and LA is illustrated in Figure 2.2a,b. In both trials, the severity of RA and LA was related in a bimodal fashion: a high severity of LAwas coupled with a high severity of RA, but not the other way around. Figure 2.2c,d illustrates the relationship between the severity of airsacculitis and systemic lesions. In both trials, the severity of systemic lesions increased with increasing severity of airsacculitis. Figure 2.2e,f shows the relationship between the severity of pericarditis and perihepatitis. In both trials, the severity of pericarditis and perihepatitis was related in a bimodal fashion: a high severity of perihepatitis was coupled with a high severity of pericarditis, but not the other way around.

2.3.3 Growth

At days 1, 4 and 7 (before the inoculation), the mean body weight of the control group was between 2% higher and 4% lower than that of the challenge group in trial 1 and between 0 and 3% lower than that of the challenge group in trial 2 ($P < 0.100$).

Figure 2.3a (trial 1) and Figure 2.3b (trial 2) show the body weight trends of the control and challenge group post inoculation. There was a lower body weight trend in

the challenge group than in the control group. Table 2.2 presents the growth retardation in the challenge group. The final growth retardation (at 14 days of age) was 11% in trial 1 and 8% in trial 2.

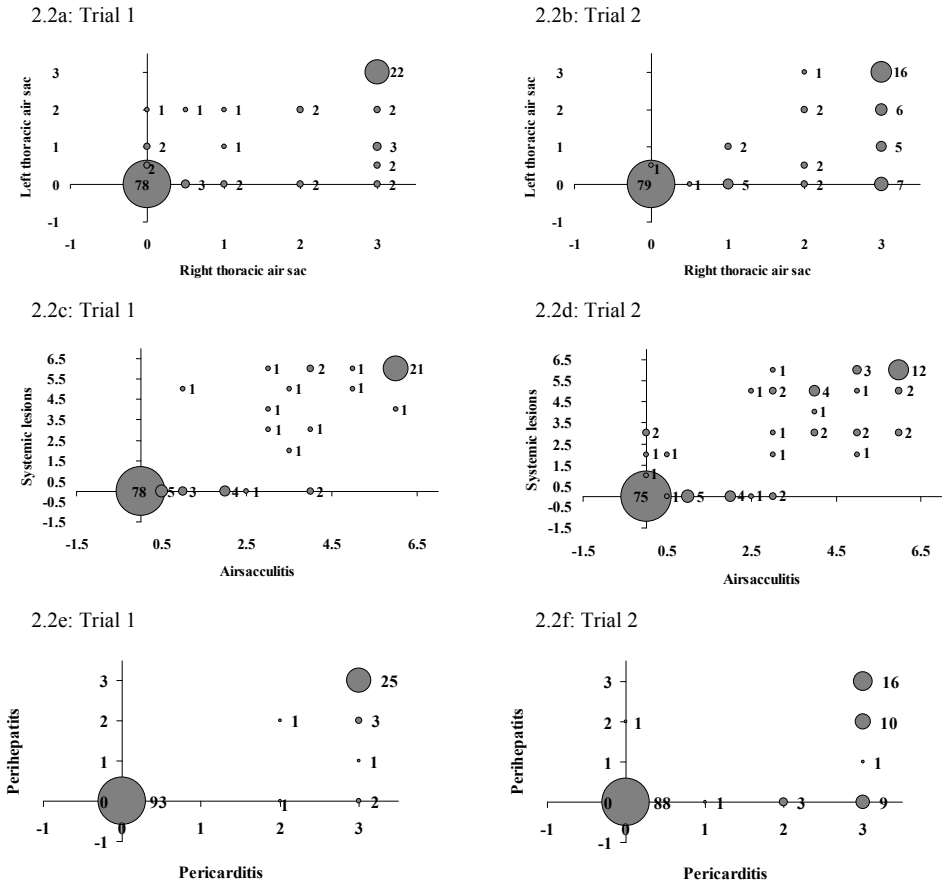

Figure 2.2. *Relationships between the severity of different types of lesions in broiler chicks surviving after challenge with E. coli. 2a, 2b: Relationship between the severity of lesions in the right and left thoracic air sac. 2c, 2d: Relationship between the severity of airsacculitis (lesions in the right and left thoracic air sac) and systemic lesions (pericarditis and perihepatitis). 2e, 2f: Relationship between the severity of pericarditis and perihepatitis.*

Figure 2.3c (trial 1) and Figure 2.3d (trial 2) illustrate the body weight trends post inoculation for the control group and the challenge group divided into subgroups according to the presence and type of lesions. The body weight trends of the chicks with systemic lesions and the chicks that died during the experiment were generally lower than that of the control group. The body weight trend for the chicks without lesions, and with airsacculitis but no systemic lesions, was almost identical to that for the control

group. Table 2.2 presents the average growth retardation in the challenge group divided into subgroups according to the presence and type of lesions. The average growth retardation of the chicks without lesions, and with airsacculitis but no systemic lesions, was not significant at any age, except for that of the chicks with airsacculitis but no systemic lesions at 14 days of age in trial 2. The average growth retardation of the chicks with systemic lesions increased with time post inoculation: from 12% at 10 days of age ($P < 0.001$) to 34% at 14 days of age ($P < 0.001$) in trial 1, and from 9% at 10 days of age ($P = 0.001$) to 25% at 14 days of age ($P < 0.001$) in trial 2. The growth retardation of the chicks that died during the experiment increased with time post inoculation, resulting in an average growth retardation at 12 days of age of 33% in trial 1 and 29% in trial 2.

Table 2.2. *Growth retardation (%) of broiler chicks challenged with E. coli relative to control group in two trials*

	Growth retardation					
Days of age:	10		12		14	
Trial:	1	2	1	2	1	2
Challenge	5.1*	3.2*	8.2*	3.1*	10.5*	7.6*
Without lesions[a]	2.5†	-0.5†	3.4†	-2.6†	1.9†	-1.9†
Airsacculitis[b]	3.4†	8.5†	5.0†	7.8†	3.0†	9.8*
Systemic lesions[c]	11.5*	8.5*	20.0*	13.5*	34.4*	25.1*
Dead	21.0*	16.5*	32.7*	29.1*	ND	ND

Growth retardation = $(\overline{BWage}_{cont} - \overline{BWage}_{chal}) / \overline{BWage}_{cont} \times 100$, where $\overline{BWage}_{cont}$ and $\overline{BWage}_{chal}$ are the body weights at 10, 12 or 14 days of age in the control- and challenge group. Results are given both in total and after grouping according to presence and type of lesions. Observations on body weights of chicks that died during the experiment are included. *Significant ($P < 0.050$) and † nonsignificant ($P < 0.100$) growth retardation. ND, not done.

[a] The surviving chicks without lesions.

[b] The surviving chicks with lesions in right and/or left thoracic airsac, but no pericarditis or perihepatitis.

[c] The surviving chicks with pericarditis and/or perihepatitis.

2.3.4 Feeding behaviour

Table 2.3 presents the proportion of chicks that did not show feeding behaviour in the control and challenge groups, in total and divided into sub-groups according to the presence and type of lesions. At 6 days of age, there was no significant difference between the control and challenge groups in the proportion of chicks that did not feed. At 11 and 13 days of age, the proportion of chicks that did not feed was between 11 and 18% higher in the challenge group than in the control group. In most cases, the proportion of chicks that did not feed in the control group was similar (not significantly different) to the group without lesions, or with airsacculitis but no systemic lesions. In contrast, the proportion of chicks that did not feed in the control group was in most cases much smaller than in the group with systemic lesions or that died during the experiment (between 27.3 and 60.9%; all significant with $P < 0.05$).

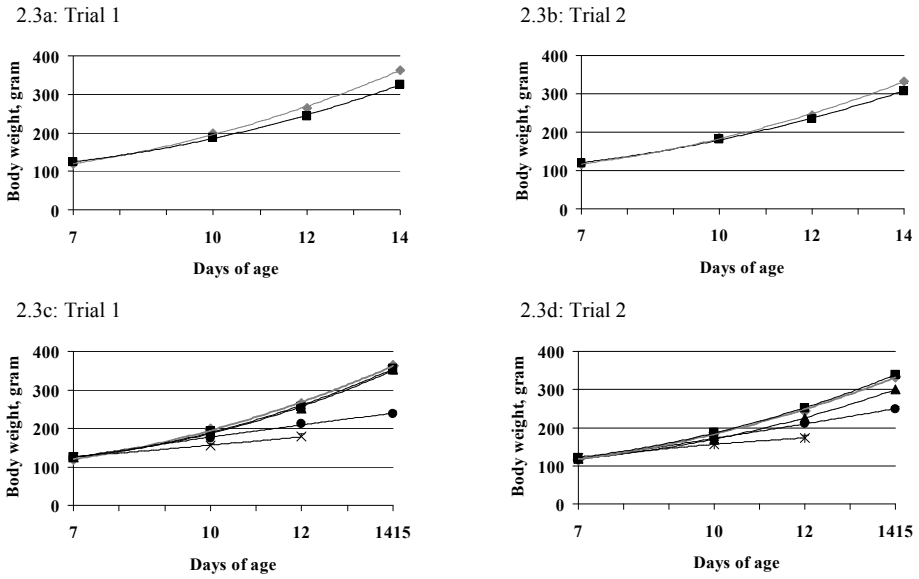

Figure 2.3. *Body weight trends of broiler chicks challenged with E. coli based on the mean body weight (g) at 7, 10, 12 and 14 days of age. 3a, 3b: In the control group (♦) and the challenge group (■). 3c, 3d: In the control group (♦) and in challenged birds grouped according to the presence and type of lesions (■, no lesions; ▲, airsacculitis only; ●, systemic lesions; ×, chicks that died). Standard errors, indicated by vertical bars, are too small to be seen at this scale. Observations on chicks that died during the experiment are included.*

Table 2.3. *Prevalence (%) of broiler chicks, challenged with E. coli in two trials, that did not show feeding behaviour 6 days of age 11 days of age 13 days age.*

Days of age	6	11		13	
	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Control	15.9^{A}	13.5^{A}	10.3^{A}	12.9^{A}	5.8^{A}
Challenge, total	18.3^{A}	26.1^{B}	21.4^{B}	26.9^{B}	23.7^{B}
Challenge:					
Without lesions	-	16.7^{A}	12.0^{A}	15.6^{A}	6.7^{A}
Airsacculitis only	-	13.3^{A}	7.7^{B}	20.0^{B}	7.7^{A}
Systemic lesions	-	42.4^{C}	12.2^{A}	45.2^{C}	51.2^{C}
Dead	-	40.8^{C}	57.1^{C}	63.6^{D}	66.7^{C}

Results are given both in total and after grouping according to presence and type of lesions; Observations on chicks that died during the experiment are also included. Different uppercase letters indicate significant ($P < 0.05$) differences between rows.

2.4 Discussion

In the present study, the responses for mortality, lesions and growth retardation were reproducible as the differences between trials were small. The response in feeding behaviour, an increase in the prevalence of chicks not feeding from category 1 to 4, was also reproducible.

The observed mortality was assumed to be due to colibacillosis because of the macroscopic *E. coli* pathology, and recovery of *E. coli* from the spleen. The prevalence of systemic lesions and mortality in the present study were both high relative to natural

colibacillosis (Matthijs et al., 2003), which is probably related to the relatively high inoculation dose and volume and the procedure (intratracheal inoculation rather than aerosol exposure) (Maatman et al., 1993; Peighambari et al., 2000). This discrepancy might appear to be in conflict with a meaningful definition of susceptibility to colibacillosis, but it actually does not pose a problem since it is the type and combination of responses (typical clinical signs or not) that matter rather than the magnitude of the response on a population level.

The surviving chicks without lesions did not show any growth retardation (Table 2.2 and Figure 2.3c,d) or reduce their feeding behaviour (Table 2.3). It is therefore reasonable to assume that these chicks reflected a category of the lowest susceptibility to colibacillosis.

The group of chicks that died due to colibacillosis showed the highest level of growth retardation (where measurable; Figure 2.3c,d), and they also reduced their feeding behaviour considerably (Table 2.3). This situation categorized the highest susceptibility to colibacillosis. There were also indications that there were differences in susceptibility among the chicks that died. The mortality occurred in one or two peaks (Figure 2.1), in accordance with previous studies (Ardrey et al., 1968; Matthijs et al., 2003), and is probably related to the cause of death. The mortality peak observed at 8 days of age in trial 1 was assumed to be associated with acute septicaemia, which is characterized by *E. coli* in the spleen, because *E. coli* was indeed recovered from the spleens and no macroscopic *E. coli* pathology was observed. The mortality peak between 11 and 13 days of age was assumed to be associated with fibrinous polyserositis, because of the presence of lesions. Acute septicaemia may reflect higher susceptibility than does fibrinous polyserositis, because of the earlier time of death. Such ranking is not necessarily valid, however, because the causes of death may reflect different types of susceptibility rather than different magnitudes. Possible differences in susceptibility between acute septicaemia and fibrinous polyserositis could not be supported by differences in growth retardation or feeding behaviour, because neither could be measured in the chicks dying of acute septicaemia. Subdivision of mortality into two categories of different susceptibility could therefore not be supported.

The surviving chicks with gross lesions clearly reflected categories of intermediate susceptibility, but the ranking among chicks with different types or severity of lesions was not obvious. The most severe lesion scores (3) were associated with the presence of systemic lesions, because this score was virtually absent in chicks that showed airsacculitis but no systemic lesions (Table 2.1). This indicates, as previously suggested by Praharaj et al. (1996), that systemic lesions reflect a higher susceptibility to colibacillosis than airsacculitis. High lesion scores were also given for airsacculitis

(Figure 2.2a,b), but these were also associated with systemic lesions (Figure 2.2c,d), and it therefore cannot be excluded that severe lesions of any type reflect a severity equal to that of systemic lesions. Support for categorizing chicks with systemic lesions as being more susceptible than chicks with airsacculitis but no systemic lesions was clearly provided for by the differences in growth retardation and in feeding behaviour between the two groups (Tables 2.2 and 2.3, and Figure 2.3c,d). Therefore, it was considered reasonable to assume that the chicks with airsacculitis but no systemic lesions reflected a category of low, intermediate susceptibility to colibacillosis, and that the chicks with systemic lesions reflected a category of high, intermediate susceptibility.

The absence of growth retardation in the chicks without lesions and those with airsacculitis but no systemic lesions is important because it suggests that, despite the general finding that colibacillosis leads to growth retardation, growth retardation is not inevitably linked to challenge with *E. coli*. In theory, all chicks infected with *E. coli* are expected to mount an immune response, which results in reduced appetite, and thereby decreased feed intake and energy availability for growth. An immune response will also cause reallocation of resources from body reserves towards defence mechanisms (Klasing & Johnstone, 1991; Sonti et al., 1996).

There are at least two plausible explanations for why the chicks without lesions and the chicks with airsacculitis but no systemic lesions showed neither reduced feeding behaviour nor growth retardation. The first explanation is that these chicks did not elicit an immune response, or release cytokines at concentrations reaching a level that inhibits feeding behaviour (Sonti et al., 1996). The presence of maternal antibodies or physical factors (e.g. ciliary activity, mucus barrier) may have provided sufficient protection in these chicks rendering an immune response superfluous. Differences in maternal antibody level are probable in the present study, because the parent stock of one of the genotypes was kept in a different environment and was 20 weeks older than the others, and chicks from older parents are expected to have more maternal antibodies (Jeurissen et al., 2000; Parmentier et al., 2004). The second plausible explanation is that these chicks elicited an immune response, but their satiety and hunger mechanisms were malfunctioning and/or they continued to preferentially allocate energy towards growth rather than towards defence mechanisms. This has previously been suggested to occur in broilers (Denbow, 1994; Qureshi & Havenstein, 1994; Reddy et al., 2002). In order to confirm or reject these possibilities, it would be necessary to investigate immunological parameters in such chicks.

In conclusion, susceptibility to colibacillosis could be meaningfully defined in four categories with increasing susceptibility: chicks without lesions, chicks with airsacculitis but no systemic lesions, chicks with (severe) systemic lesions, and chicks that died.

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Chapter 3: Genetic Variation among Broiler Genotypes in Susceptibility to Colibacillosis

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Abstract: Selection for reduced susceptibility to colibacillosis in broilers may contribute to the prevention of colibacillosis. Such selection should focus on the responses to *E. coli* rather than the associated primary agent(s). The purpose of the current study was to examine whether genetic variation is present in the susceptibility to colibacillosis. This was achieved through an evaluation of the susceptibility to primary colibacillosis in 5 pure broiler lines, a slow-growing line, and two 2-way crosses of the pure lines (altogether referred to as genotypes). A challenge experiment was executed in 2 trials. Per trial, 24 chicks per genotype were challenged and 20 chicks per genotype were controls. At 7 d of age, challenged chicks were intratracheally inoculated with 0.3 mL of *E. coli* O78K80 solution, and controls with 0.3 mL of PBS. All chicks were euthanized at 14 or 15 d. Traits measured were mortality, lesion scores (airsacculitis, pericarditis, and perihepatitis) at 14 or 15 d, and BW at 1, 4, 7, 10, 12, and 14 or 15 d. An effect of genotype on mortality, lesion prevalence, and growth retardation was found, indicating the presence of genetic variation in susceptibility to colibacillosis, and suggesting that selection for reduced susceptibility is possible. There were large between-genotype differences in mortality (up to 46%) and in lesion prevalence (up to 41%). Growth retardation was not observed for any genotype in chicks without lesions, whereas genotypes differed from none to 20% growth retardation for chicks with airsacculitis but no systemic lesions, and up to 13% for chicks with systemic lesions. The heterosis in susceptibility and growth retardation was found to be either negative or absent, indicating that crossbreeding would not be an advantage for the selection for reduced susceptibility, and that test crossing is essential.

***Keywords**: Genetic Variation, Growth Retardation, Lesion, Colibacillosis, Broiler*

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3.1 Introduction

Exploitation of genetic variation in the improvement of disease resistance in commercial poultry breeding has so far been limited to a few specific diseases; for example, Marek's disease, leucosis, and ascites, and to survival in general (Gavora, 1998; Kreager, 1998; McKay et al., 2000). To the authors' knowledge, there is no published evidence of successful selection for resistance to specific bacterial diseases, such as colibacillosis (fibrinous polyserositis). Colibacillosis, which predominantly occurs as a secondary infection after 3 wk of age but also as a primary infection at younger age (Goren, 1978), has long been recognized as an economically important disease in broilers, mainly because of its adverse effects on growth (Ardrey et al., 1968; Goren, 1991; Vandemaele et al., 2002).

Selection for reduced susceptibility to colibacillosis may contribute to its prevention, because improved hygiene, vaccination, and the use of therapeutics are insufficiënt in providing complete prevention of colibacillosis (Goren, 1991). Genetic variation is present in mortality, lesions, and antibody response to *E. coli* challenge with heritabilities for antibody response ranging from 0.21 to 0.72 (Pitcovski et al., 1987; Bumstead et al., 1989; Leitner et al., 1992; Praharaj et al., 1996b). However, these studies have focused on susceptibility to the primary agent(s) associated with colibacillosis (e.g., infectious bronchitis virus and Newcastle disease) rather than to *E. coli* as such. Whether the primary agent(s) or *E. coli* itself is the main cause of common responses [mortality, airsacculitis, and polyserositis (i.e., pericarditis and perihepatitis)] in secondary colibacillosis is unclear (Bumstead et al., 1989; Gross, 1990; Peighambari et al., 2000). It is, however, clear that the only certain common denominator in colibacillosis is the *E. coli* bacterium; therefore, selection for reduced susceptibility to colibacillosi should focus on the responses to *E. coli* rather than the associated primary agent(s).

The purpose of the current study was to examine whether genetic variation is present in susceptibility to colibacillosis. This was achieved through an evaluation of the responses in mortality, airsacculitis, systemic lesions, and growth retardation to an *E. coli* challenge in 5 pure broiler lines, a slow-growing line, and two 2-way crosses of the pure lines.

3.2 Materials and Methods

3.2.1 Chicks

Eggs were incubated and hatched in 2 trials at the Spelderholt Poultry Research Center in Beekbergen, The Netherlands. The eggs originated from 5 pure broiler lines: 2 sire (sire1 and sire2), 3 dam (dam1, dam2, and dam3), 1 slow-growing line (SlowGrow), and

2 crosses: a sire and a dam cross (SireCross and DamCross). Table 3.1 shows the universal line codes. Henceforth, the lines and crosses altogether will be referred to as genotypes. At the time of egg collection, the age of the parent stock of the slow-growing line was 47 wk (trial 1) or 50 wk (trial 2), whereas the age of the parent stocks for the other genotypes was 27 wk (trial 1) or 30 wk (trial 2).

Table 3.1. The mortality and lesion prevalences (%) per broiler genotype, based on the number of chicks per genotype that were challenged (lesions were not scored on the chicks that died during the experiment)

Genotype[1]	n at 7 d	Mortality	Lesions		
			Airsacculitis[2]	Systemic lesions[3]	Total
SlowGrow (3)	43	0.0[a]	11.6[ab]	41.9[a]	53.5[a]
Dam1 (A3)	44	25.0[b]	4.6[cd]	15.9[bc]	20.5[bc]
Dam2 (E4)	46	43.5[c]	4.4[cd]	21.7[bd]	26.1[b]
Dam3 (E5)	44	45.5[c]	6.9[ac]	22.7[bd]	29.6[b]
Sire1 (A2)	45	26.7[b]	2.1[d]	10.9[ce]	13.0[d]
Sire2 (E3)	44	22.7[b]	4.6[cd]	13.6[ce]	18.2[cd]
DamCross (A3×E4)	44	31.8[bc]	13.6[b]	27.3[d]	40.9[a]
SireCross (A2×E3)	46	43.5[c]	8.7[abc]	8.7[e]	17.4[cd]

[a-e]Percentages within a column lacking a common superscript differ ($P < 0.05$).

[1]SlowGrow = a slow-growing line; Dam1, Dam2, and Dam3 = dam lines; Sire1 and Sire2 = sire lines; and DamCross and SireCross = crosses between Dam1 and Dam2 and between Sire1 and Sire2, respectively.

[2]The surviving chicks with lesions in right or left thoracic airsac or both, but no pericarditis or perihepatitis.

[3]The surviving chicks with pericarditis, perihepatitis, or both.

3.2.2 Experimental Design

At 1 d of age (at hatch), the chicks were individually tagged with badges and divided into a challenge and a control group. In both groups there were 4 pens. In each trial, there were 192 chicks in the challenge group (48 chicks per pen), and 160 chicks in the control group (40 chicks per pen). There were more chicks in the challenge group than in the control group to anticipate losses due to mortality. Genotype and sex were equally represented. In each pen in the challenge group, there were 3 males and 3 females per genotype. In the control group, there were 3 males and 2 females per genotype in each of 2 of the 4 pens, and 2 males and 3 females per genotype in the other 2 pens.

The challenge and the control group were kept in separate, but identical, climate-controlled cells to avoid horizontal infection of control chicks. The pens in both groups each covered an area of 1.54 × 1.75 m, had walls that were 0.5 m high, and were provided with litter in the form of sawdust. Feed and water was provided ad libitum. The feed was a commercial standard mix for starters with 12.77 MJ of ME/kg and 20.8% CP. A schedule of 20L:4D was practiced, with lights on at 0600 h. The temperature followed a standard schedule, starting at 34°C at 1 d of age, followed by a gradual decline to 24°C at 15 d of age. The humidity was kept at 50%.

3.2.3 Challenge

At 7 d of age, all chicks in the challenge group were inoculated intratracheally with a 1:100 PBS solution of an *E. coli* O78K80 (506) strain cultured in glucose peptone broth. The inoculation was done using a 1.0-mL syringe fitted with a blunt-ended pipette tip (Cat. No. 4862, Corning, Inc., New York, NY). The *E. coli* 506 strain was a flumequine-resistant strain isolated from an inflamed pericardium of a commercial broiler suffering from natural colibacillosis (van Eck and Goren, 1991). In the first trial, 25 chicks from 1 pen were inoculated with 0.5 mL of *E. coli* solution with a total of $10^{6.3}$ cfu, but 4 of these chicks showed signs of suffocation within 15 min postinoculation. For the remainder of the chicks, the volume was therefore adjusted to 0.3 mL with $10^{6.0}$ cfu in trial 1 and $10^{5.8}$ cfu in trial 2. All chicks in the control group were inoculated intratracheally with 0.3 mL of PBS.

Chicks that died during the experiment were dissected and a macroscopic examination was done. Lesions were not scored. A bacteriological examination of the spleen of all chicks that died during the experiment was performed, and the sensitivity of the *E. coli* isolates was compared with that of the *E. coli* 506 strain as described by Velkers et al. (2005). The cause of death was considered to be colibacillosis if there were signs of airsacculitis, pericarditis, or perihepatitis, or if the *E. coli* strain 506 could be isolated from the spleen. According to this definition, there was one chick that did not die due to colibacillosis.

The chick that died of a cause other than colibacillosis was omitted from the analyses, as were the 25 chicks that received 0.5 mL of *E. coli* inoculate and the 9 chicks that died before inoculation (6 in the challenge group and 3 in the control group). The number of chicks per genotype in the challenge group that were included in the analysis is given in Table 3.1 (n at d 7). From the control group, between 37 and 40 chicks per genotype were included in the analysis.

3.2.4 Recording of Traits

Mortality was recorded every morning. The surviving chicks were stunned by electrocution and euthanized by bleeding: one-half of the challenge and control groups were euthanized at 14 d, and the other half at 15 d. The following lesion types were scored macroscopically: airsacculitis (right and left thoracic air sac), pericarditis, and perihepatitis. Airsacculitis was considered representative of *E. coli* pathology of the respiratory tract, whereas pericarditis and perihepatitis were considered representative of systemic *E. coli* pathology. Lesion scoring was performed as described by van Eck and Goren (1991) using the following scale: 0 = no lesions, 0.5 = one yellow or brown pinhead-sized spot indicative of inflammation, 1 = two or more pinhead-sized spots

indicative of inflammation, 2 = thin layer of fibrinous exudate on various locations, and 3 = thick and extensive layer of fibrinous exudate. The scoring was carried out blind with respect to both challenge and genotype, except for scoring of the SlowGrow line, which was recognizable due to its color.

The BW was recorded 6 times during the experiment. Chicks were individually weighed at 1, 4, 7, 10, and 12 d, and one-half of the challenge and control pens was weighed at 14 d and the other half at 15 d.

3.2.5 Trait Definitions

Mortality was defined as the mortality in the challenge group postinoculation as the percentage of total number of chicks at 7 d. Airsacculitis was defined as the prevalence of lesions in right or left thoracic air sac or both relative to the number of challenged chicks. Systemic lesions were defined as the prevalence of pericarditis or perihepatitis or both relative to the number of challenged chicks. Lesion prevalence was defined as airsacculitis or systemic lesions or both. Susceptibility to colibacillosis was defined as a categorical trait with 4 categories of increasing susceptibility (Ask et al., 2006): 1) chicks without lesions, 2) chicks with airsacculitis but no systemic lesions, 3) chicks with systemic lesions, and 4) chicks that died during the experiment.

The BW at 1, 4, 7, 10, and 12 d of age were designated BW1, BW4, BW7, BW10, and BW12, respectively. The BW at 14 or 15 d was treated as one trait, designated BW14. In the analyses of BW, the observations on chicks that died during the experiment were also included. Growth retardation was defined as: $(BW14_{cont} - BW14_{chal})/BW14_{cont} \times 100$; where $BW14_{cont}$ and $BW14_{chal}$ were the least squares means of the BW at 14 d in the control and challenge groups. In the analysis of growth retardation, the observations on chicks that died during the experiment were omitted.

3.2.6 Data Analysis

The Kruskal-Wallis test was used to test for differences in mortality and lesion prevalence between genotypes. When sample sizes were not sufficiently large, the Pearson's χ^2 test was used. An ANOVA (F-test) was used to test for the effect of genotype on BW depending on treatment (challenge or control group), and the t-test was applied to test for the effect of treatment on BW depending on genotype. Tukey's adjustment (the Tukey-Kramer method) was used to correct for multiple comparisons. The model was:

$$Y_{ijklmn} = \mu_{ijklmn} + \text{TRIAL}_i + \text{TREATMENT}_j + \text{SEX}_k + \text{GENOTYPE}_l + \text{DAY1415}_m \text{ (for BW14)}$$

$$+ \text{TREATMENT}\times\text{GENOTYPE}_n + e_{ijklmn},$$

where Y_{ijklmn} = the BW in the ith trial, the jth treatment, the kth sex, the lth genotype, the mth DAY1415, and the nth interaction between treatment and genotype, DAY1415 = the age at which the final measurement of BW was taken, and e_{ijklmn} = the random residual effect.

The *t*-test was applied to test for the effect of susceptibility on BW depending on genotype. Tukey's adjustment (the Tukey-Kramer method) was used to correct for multiple comparisons. The model was:

$$\begin{aligned} Y_{ijklmn} &= \mu_{ijklmn} + \text{TRIAL}_i + \text{TREATMENT}_j + \text{SEX}_k \\ &+ \text{GENOTYPE}_l + \text{DAY1415}_m \text{ (for BW14)} \\ &+ \text{SUSCEPTIBILITY}\times\text{GENOTYPE}_n + e_{ijklmn}, \end{aligned}$$

where Y_{ijklmn} = the BW in the ith trial, the jth treatment, the kth sex, the lth genotype, the mth DAY1415, and the nth interaction between susceptibility (control group and susceptibility in the challenge group) and genotype, and e_{ijklmn} = the random residual effect.

The *t*-test was applied to test for differences in growth retardation, using Bonferroni's adjustment to correct for multiple comparisons. Heterosis was defined as the difference between the cross and the mean of the 2 parent lines (Falconer and Mackay, 1996).

3.2.7 Ethics

The experiment was approved by the Animals Ethics Committee (Dierexperimentencommissie, Utrecht University, The Netherlands), and chicks were handled accordingly. The Animal Ethics Committee based its decision on the *Wet op Dierproeven* (1996) and on the *Dierproevenbesluit* (1985); both are available online (http://www.nca-nl.org/).

3.3 Results and Discussion

3.3.1 Mortality and Lesion Prevalence

In Table 3.1, the prevalence of chicks with different susceptibilities to colibacillosis is given per genotype. There was an effect of genotype on mortality ($P < 0.001$), and differences were large (up to 46%). The Dam3, the Dam2, and the SireCross genotypes showed the highest mortality, whereas the SlowGrow line did not show any mortality. There was also an effect of genotype on airsacculitis ($P = 0.001$) and systemic lesions ($P = 0.046$), and differences between genotypes were large (up to 41% in total). The SlowGrow, Dam2, Dam3, and SireCross lines showed the highest lesion prevalences,

whereas the Sire1 and the Sire2 showed the lowest. Genotype differences were therefore apparent, which indicates the presence of genetic variation in susceptibility to colibacillosis. This suggests that selection for reduced susceptibility to colibacillosis is possible, and selection may therefore contribute to the prevention of colibacillosis in broilers. Within-line selection would first require the estimation of genetic parameters, however, and there was no pedigree available in the present study to enable this. The estimation of genetic parameters for even one single genotype would require challenging a large number of pedigreed chicks, and due to the expected detrimental consequences of the *E. coli* challenge, justification would have been very difficult without having shown beforehand that genetic variation is present.

The relative responses of the SlowGrow line are in agreement with previous studies in which relatively slow growing experimental lines have been observed to have lower mortality but higher lesion prevalence than commercial broilers (Yunis et al., 2002). The apparent lower susceptibility of these lines may be due to differences in maternal antibodies. The experimental lines may have had a relatively high amount of maternal antibodies simply due to the procedure of selecting for high and low antibody response to *E. coli* vaccination. The vaccination was done at an early age where maternal antibodies may have been present; that is, selecting for a low response may actually be equal to selecting for a high amount of maternal antibodies. In the present study, the age of the parent stock of the SlowGrow line may have contributed to a relatively high amount of maternal antibodies, because chicks from older parents are expected to have more maternal antibodies (Jeurissen et al., 2000; Parmentier et al., 2004).

The specific ranking of the genotypes should be interpreted with care because it may be influenced by the experimental design. For instance, the duration of the experiment might influence the ranking of genotypes. The duration of the challenge (7 or 8 d) was based on the expectation, in accordance with previous research with commercial broilers (J. H. H. van Eck, Utrecht Univ., Utrecht, The Netherlands, unpublished data), that the highest prevalence of, and variation in, lesions would occur around 7 d postchallenge. This duration was therefore expected to be optimal for the evaluation of genotype differences, although it is possible that the optimal duration for evaluation differs among genotypes. Some genotypes may, on average, already have been in recovery, whereas others may, on average, have still been deteriorating. For example, it is possible that a proportion of the SlowGrow chicks with lesions would have died if the experiment duration had been longer. In fact, a closer examination of the lesion scores revealed that the proportion of chicks with lesions, and at least one lesion score of the highest severity, was higher in the SlowGrow (35%) than in the Dam1, Sire1, and Sire2 genotypes (between 12 and 21%).

3.3.2 BW

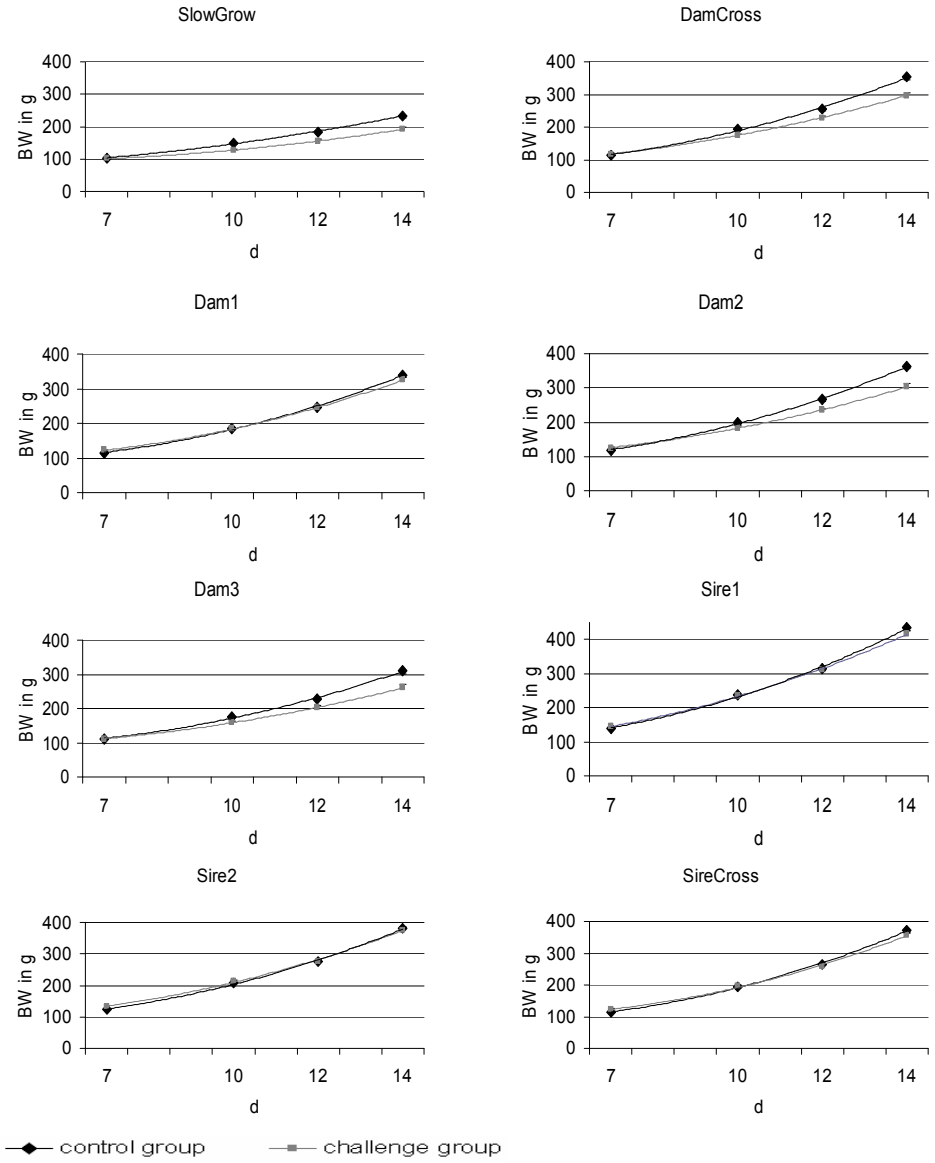

Figure 3.1. Body weight trends postinoculation (7 d) of the different broiler genotypes and crosses based on the least squares means of BW at 7, 10, 12, and 14 d. Vertical bars indicate SE. Observations on chicks that died during the experiment were included.

The interaction between treatment (challenge or control group) and genotype was significant for BW after challenge: BW10 ($P < 0.001$), BW12 ($P < 0.001$), and BW14 (P

= 0.002), but not before challenge: BW1 (P = 0.187), BW4 (P = 0.089), and BW7 (P = 0.053), suggesting a genotypespecific response to challenge in BW. The genetic variation among genotypes in response to challenge also indicated the presence of genetic variation in coping with an *E. coli* challenge, which is in agreement with previous studies (Maatman et al., 1993; Praharaj et al., 1996b; Cheng et al., 2004).

The specific ranking of the genotypes may be specific to the experimental design (e.g., depending on age at and duration of challenge). In Figure 3.1, the BW trends in the challenge and control groups per genotype are given. The BW trends of the Dam1, Sire1, Sire2, and SireCross genotypes did not differ between the control and challenge groups. This appears to be a very interesting result for commercial broiler breeding, because certain genotypes do not show growth retardation at all, and genotypes that have been highly selected for growth are even among these genotypes. However, it may also simply be a result of the inclusion of chicks that had lesions or died during the experiment, because these chicks showed the most growth retardation (Ask et al., 2006) and both mortality and lesion prevalence differed per genotype. In genotypes in which the mortality and systemic lesion prevalence were lower, the overall growth retardation of the challenge group would be expected to be lower as well, and this was indeed observed.

Table 3.2. Growth retardation[1] (%) per broiler genotype at 14 d in the challenge group, divided in groups according to presence and type of lesions (susceptibility to colibacillosis).

Genotype (code)[2]	Growth retardation		
	Without lesions[3]	Airsacculitis[4]	Systemic lesions[5]
SlowGrow (3)	6.5^{a}	15.2^{ab}	31.5^{a}
Dam1 (A3)	-3.7^{a}	-0.2^{c}	32.9^{a}
Dam2 (E4)	3.2^{a}	19.5^{b}	34.3^{a}
Dam3 (E5)	2.7^{a}	15.3^{ab}	31.5^{a}
Sire1 (A2)	-1.5^{a}	3.2^{c}	35.6^{a}
Sire2 (E3)	-2.8^{a}	-11.4^{ad}	22.5^{b}
DamCross (A3×E4)	0.5^{a}	13.8^{a}	32.2^{a}
SireCross (A2×E3)	1.7^{a}	-7.9^{cd}	25.6^{b}

$^{a-d}$Growth retardation within a column lacking a common superscript differ ($P < 0.05$).

[1]Growth retardation = $(BW14_{cont}–BW14_{chal})/BW14_{cont} \times 100$; where $BW14_{cont}$ and $BW14_{chal}$ are the BW at 14 d in the control and challenge groups, respectively. Observations on chicks that died during the experiment were not included.

[2]SlowGrow = a slow-growing line; Dam1, Dam2, and Dam3 = dam lines; Sire1 and Sire2 = sire lines; and DamCross and SireCross = crosses between Dam1 and Dam2 and between Sire1 and Sire2, respectively.

[3]The surviving chicks without lesions.

[4]The surviving chicks with lesions in right or left thoracic air sac or both, but no pericarditis or perihepatitis.

[5]The surviving chicks with pericarditis, perihepatitis, or both.

In Table 3.2, the growth retardation per genotype depending on the susceptibility to colibacillosis is given. The chicks without lesions did not show growth retardation in any genotype. The chicks with airsacculitis but no systemic lesions did not show growth retardation in the Dam1, the Sire1, and the SireCross lines, whereas growth retardation

in the other genotypes was between 11 and 20%. The chicks with systemic lesions showed high growth retardation in all genotypes, differing up to 13% between genotypes, indicating that the genotypes coped differently with colibacillosis.

There are at least 2 plausible explanations for such differences in coping style: 1) not eliciting an immune response, which results in reduced appetite or resource allocation toward the immune response, and 2) allowing for prioritization of growth along with an immune response through alterations in regulation of feed intake (Leenstra, 1992; Coop and Kyriazakis, 1999). Both mechanisms would enable broilers to acquire sufficient energy for both growth and immune response.

The implications for commercial breeding of the first explanation for the differences in coping style include that at older age, the susceptibility to colibacillosis could be differently expressed, and possibly result in different ranking of genotypes. For example, maternal antibodies could be present at sufficiently high levels to prevent an immune response in young chicks, but not in older chicks, and the expressed susceptibility to colibacillosis would probably change.

The implications for commercial breeding of the second explanation include unknown consequences of long-term intensive selection for broiler production traits. Klasing and Johnstone (1991) have suggested that chicks reduce feed intake in relation to an immune response, but the regulation of feed intake in broilers, especially shortly after hatch, has been altered due to selection for BW (Barbato, 1994; Denbow, 1994; Bokkers and Koene, 2003). The possibility of regulatory alterations allowing for increased feed intake, however, appears to be questionable. High BW-selected broilers have been suggested to already eat to their full capacity (Nir et al., 1978; Barbato et al., 1984), and the growth rate of broilers less than 3 wk old is thought to be restricted by the capacity to ingest and digest feed (Lilja et al., 1985; Denbow, 1994). In contrast, regulatory alterations allowing for improved feed efficiency, such as behavioral strategies of different metabolic costs or allocation of resources to, for instance, visceral organs rather than muscle and fat tissue, may very well be achieved (Lilja et al., 1985; Benson et al., 1993; Shapiro and Nir, 1995; Deerenberg and Overkamp, 1999).

Chicks that are already highly efficient, however, may be less likely to be capable of further improving feed efficiency during immunological challenge (van Eerden et al., 2004). The genotypes, which are expected to be the most efficient when nonchallenged (Sire2 and Dam2 with average feed conversion ratios of 1.68 and 1.70), would therefore not be expected to be capable of improving efficiency further during challenge to prevent growth retardation. The expected most efficient genotype, when nonchallenged (Sire2), showed low growth retardation, however, and the expected least-efficient genotype, when nonchallenged (the SlowGrow), showed high growth retardation. Thus,

if an immune response was elicited, either selection for improved feed efficiency has not impaired the ability of chicks to divert resources toward an immune response, or the immune response did not result in an energy demand, which could not be supported by the feed intake.

3.3.3 Heterosis

Both crosses showed either no or negative heterosis for all responses to the *E. coli* challenge. The DamCross showed negative heterosis with regards to total lesion prevalence (−17.6%, $P < 0.05$). The SireCross showed negative heterosis with regards to mortality (−18.8%, $P < 0.05$), and with regards to the prevalence of chicks with airsacculitis but no systemic lesions (−5.35%, $P < 0.05$).

The absence of (positive) heterosis in the 2 crosses for most responses to the *E. coli* challenge, and the presence of negative heterosis for lesion prevalence indicated that crossbreeding would not provide any advantage for selection for reduced susceptibility to colibacillosis. Previously, heterosis in lesion prevalence in response to *E. coli* challenge was observed to vary from none to 27% (Praharaj et al., 1996a), which is in disagreement with the negative heterosis observed in the present study. Both the observation of negative heterosis and the disagreement with previous studies stress the importance of test crossing in broiler breeding. Even in a fitness-related trait like susceptibility to colibacillosis, positive heterosis cannot be universally expected. In general, negative heterosis has been observed in many different traits, including fitness-related traits; and for the same traits, differing degree and sign of heterosis has been observed for different crosses (Boa-Amponsem et al., 1998; Deng and Fu, 1998; Deeb and Lamont, 2002).

In conclusion, genetic variation in susceptibility to colibacillosis and in the resulting growth retardation has been demonstrated, which suggests that selection against this susceptibility is possible. The absence of, or negative, heterosis suggests that crossbreeding will not provide any advantage for selection for reduced susceptibility to colibacillosis, but that test crossing will be essential in the identification of the best cross(es).

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Chapter 4: Role of Thyroid Hormones, Maternal Antibodies, and Antibody Response in the Susceptibility to Colibacillosis of Broiler Genotypes

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Abstract: The purpose of this study was to investigate whether differences in susceptibility to colibacillosis are associated with maternal antibodies, antibody response, and alterations in thyroid hormones [triiodothyronine (T_3) and thyroxine (T_4)] and to investigate the effect of genotype on the changes in T_3 and T_4 during challenge and antibody response. A challenge experiment was executed in 2 trials. Per trial, 24 chicks per genotype were challenged, and 20 chicks per genotype were controls. At 7 d of age, challenged chicks were intratracheally inoculated with 0.3 mL of *E. coli* O78K80 and controls with 0.3 mL of PBS. All chicks were euthanized at 14 or 15 d. Thyroid hormone plasma concentrations and *E. coli* specific antibody titers (AB) were measured at 7 d (T_{3d7}, T_{4d7}, and AB_{d7}) and 14 or 15 d (change from 7 to 14 or 15 d was analyzed: ΔT_3, ΔT_4, and ΔAB). Susceptibility was defined based on mortality, lesions, growth retardation, and eating behavior. There was a significant effect of challenge on T_{3d7}; probably due to eating pattern in association with circadian rhythm. The challenge group was suggested to have functional hypothyroidism relative to the control group, indicating metabolic changes due to the challenge, and it was indicated that an antibody response was elicited. Differences in susceptibility were not significantly related to differences in T_{3d7}, T_{4d7}, ΔT_3, or ΔT_4 or to maternal antibodies (AB_{d7}), but the antibody response tended to increase (decreasing ΔAB) with increasing susceptibility. There were indications of genetic variation in T_{4d7}, ΔT_4, AB_{d7}, and ΔAB, but there was no observed effect of genotype on ΔT_3 and ΔT_4 during challenge or on the antibody response. Further, there were indications that selection for growth traits has resulted in alterations in ΔT_4 due to challenge, as indicated by a lower ΔT_4 in the challenge group relative to the control group for more intensively selected genotypes as opposed to a higher ΔT_4 for less intensively selected genotypes.

Keywords: *Triiodothyronine, Thyroxine, Antibody Response, Broiler, Susceptibility*

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4.1 Introduction

Colibacillosis is a frequent occurring respiratory disease in broilers caused by the *E. coli* bacterium, which has adverse effects on growth and health, growth retardation being the main problem (Goren, 1991; Vandemaele et al., 2002). Recently, it has been established that even in the face of a severe *E. coli* challenge, there is considerable variation in the susceptibility to colibacillosis, as expressed by gross lesions, growth retardation, and eating behavior (Ask et al., 2006b). Moreover, it has been found that the susceptibility is subject to genetic variation (Ask et al., 2006a), which offers prospects for selection against susceptibility. However, the biological background (e.g., physical, physiological, ethological, and immunological factors) of the variation in susceptibility to colibacillosis is not yet fully understood, although this is essential to foresee potential (negative) side effects of selection. In this study, we attempted to gain insight into metabolic changes and immune response to an *E. coli* challenge and their relationship with susceptibility to colibacillosis.

In the face of a pathogenic challenge, an animal may be able to cope without showing growth retardation and eliciting an immune response if it possesses sufficient maternal or innate immunity (in the form of physical, chemical, or biological barriers) to resist the challenge. If this is not the case, however, the animal must elicit an immune response. Eliciting an immune response also involves significant metabolic changes [i.e., a general increase in metabolic energy expenditure and net proteolysis and lipolysis (Beisel, 1975)] and a reduction in appetite (Sonti et al., 1996), which, on their own or combined, result in growth retardation (Klasing and Johnstone, 1991). Improved knowledge on metabolic changes, maternal immunity, as well as on immune response could therefore provide insight into the biological background of the observed variation in susceptibility to colibacillosis. The thyroid hormones [triiodothyronine (T_3) and thyroxine (T_4)] are key elements in metabolism, affecting general metabolic rate as well as protein and adipose turnover rates (Reece, 1997), and information on the levels and changes in these hormones during challenge may therefore be a relatively informative indicator of metabolic changes during challenge. Antibody response is part of the humoral immune response but may also function as a relatively informative indicator of an immune response in general, because the humoral immune response depends on an activation of the innate immune response (Tizard, 2004).

Alternatively to coping with a pathogenic challenge through sufficient maternal or innate immunity, an altered regulation of metabolism and appetite during an infection in broilers may also allow a broiler to cope without showing growth retardation. The metabolic changes

that are normally involved in an immune response (innate or humoral) make sense from an evolutionary and a teleological point of view: For an effective immune response, the requirements for resources are usually acute, but, in the wild, obtaining feed and converting feed into energy and protein is costly and time-consuming, and the possibility to acquire external resources is decreased in an infected animal. In contrast, in an environment with ad libitum and easy accessible feed, the use of body reserves as a source of energy and protein and the cessation of eating during infection may not be the most sensible strategy. The intensive selection for growth in broilers in an environment with ad libitum and easy accessible feed may contribute to a change in coping strategy through changes in prioritization of body functions toward growth rather than "traditional" fitness traits, such as disease resistance. It is therefore hypothesized that the regulation of metabolic changes and appetite in broilers during an immune response (innate or humoral) is altered, and differences among broiler genotypes may provide insight into this matter.

The purpose of this study was 2-fold: 1) to investigate whether differences in susceptibility to colibacillosis are associated with maternal antibodies, antibody response, and alterations in T_3 and T_4 hormones; and 2) to investigate the effect of genotype on the changes in T_3 and T_4 during challenge and on antibody response.

4.2 Materials and Methods

A challenge experiment was carried out on a population consisting of multiple broiler lines and crosses to ensure a broad genetic inference of results. The susceptibility of the individual lines and crosses to colibacillosis is presented in Ask et al. (2006a).

4.2.1 Chicks

Eggs were incubated (at 37.8°C) and hatched (34°C in hatcher) in 2 trials at the Spelderholt Institute for Poultry Research in Beekbergen, The Netherlands. The eggs originated from 6 pure broiler lines: 2 sire [Sire1 (A2) and Sire2 (E3)], 3 dam [Dam1 (A3), Dam2 (E4), and Dam3 (E5)], and 1 slow-growing line [SlowGrow (3)] and 2 crosses: a sire and a dam cross [SireCross (A2 · E3) and DamCross (A3 · E4)]. Henceforward, the lines and crosses together will be referred to as genotypes. At the time of egg collection, the age of the parent stock of the slowgrowing line was 47 wk (trial 1) or 50 wk (trial 2), whereas the age of the parent stocks for the other genotypes was 27 wk (trial 1) or 30 wk (trial 2). All parent stock, except for that of the SlowGrow, was kept in groups at the pureline pedigree farm in Herveld, The Netherlands, which is foreseen with a filtered air and positive pressure system. The SlowGrow parent stock was kept at a separate commercial production unit. A total of 240 eggs were incubated

from each of the Dam1, Dam2, Dam3, DamCross, and SlowGrow genotypes. From each of the Sire1 and Sire2 genotypes, a total of 300 eggs were incubated, and from the SireCross, a total of 293 eggs were incubated. The hatchabilities of the respective genotypes were 82% (Dam1), 76% (Dam2), 81% (Dam3), 66% (DamCross), 88% (SlowGrow), 40% (Sire1), 52% (Sire2), and 58% (SireCross).

4.2.2 Experimental Design

At 1 d of age (at hatch), the chicks were individually tagged with badges and allocated randomly to a challenge and a control group, each with 4 pens. In each trial, there were 192 chicks in the challenge group (48 chicks per pen) and 160 chicks in the control group (40 chicks per pen). There were more chicks in the challenge group than in the control group to anticipate losses due to mortality. Genotype and sex were equally represented: In each pen in the challenge group, there were 3 males and 3 females per genotype. In the control group, there were 3 males and 2 females per genotype in each of 2 of the 4 pens and 2 males and 3 females per genotype in the other 2 pens.

The challenge and the control group were kept in separate but identical climate-controlled cells to avoid horizontal infection of control chicks. The pens in both groups each covered an area of 1.54 · 1.75 m, had walls that were 0.5 m high, and were provided with litter in the form of sawdust. Feed and water were provided ad libitum. The feed was a commercial standard mix for starters with 12.77 MJ of ME and 20.8% CP. A schedule of 20L:4D was practiced with lights on at 0600 h. The temperature followed a standard schedule, starting at 34°C at 1 d of age, followed by a gradual decline to 24°C at 15 d of age. The humidity was kept at 50%.

4.2.3 Challenge

At 7 d of age, all chicks in the challenge group were individually inoculated intratracheally with a 1:100 PBS of an *E. coli* O78K80 (506) strain cultured in glucose peptone broth. The inoculation was done using a 1.0-mL syringe fitted with a blunt-ended pipette tip (4862, Corning Inc., Corning, NY). The *E. coli* 506 strain was a flumequine-resistant strain isolated from an inflamed pericardium of a commercial broiler suffering from natural colibacillosis (van Eck and Goren, 1991). In the first trial, 25 chicks from 1 pen were inoculated with 0.5 mL of *E. coli* solution with a total of $10^{6.3}$ cfu, but 4 of these chicks showed signs of suffocation within 15 min postinoculation. For the remainder of the chicks, the volume was therefore adjusted to 0.3 mL, with a total of $10^{6.0}$ cfu in trial 1 and $10^{5.8}$ cfu in trial 2. All chicks in the control group were inoculated intratracheally with 0.3 mL of PBS.

Chicks that died during the experiment were dissected, and a macroscopic examination was done. Lesions were not scored. A bacteriological examination of the spleen of all chicks that died during the experiment was performed, and the sensitivity of the *E. coli* isolates was compared with that of the *E. coli* 506 strain, as described by Velkers et al. (2005). The cause of death was considered to be colibacillosis if there were signs of airsacculitis, pericarditis, or perihepatitis or if the *E. coli* strain 506 could be isolated from the spleen. According to this definition, there was 1 chick that did not die due to colibacillosis.

The chick that did not die due to colibacillosis, was omitted from the analyses, as were the 25 chicks that received 0.5 mL of *E. coli* inoculate and the 9 chicks that died before the inoculation (6 in the challenge group and 3 in the control group).

4.2.4 Recording of Traits

Mortality was recorded every morning. The surviving chicks were stunned by electrocution and euthanized by bleeding; half of the challenge and the control group were euthanized at 14 d of age and the other half at 15 d of age. The following lesion types were scored macroscopically: airsacculitis (right and left thoracic air sac), pericarditis, and perihepatitis. Airsacculitis was considered as representative for *E. coli* pathology of the respiratory tract, whereas pericarditis and perihepatitis were considered as representative for systemic *E. coli* pathology. Lesion scoring was performed as described by van Eck and Goren (1991).

Chicks were individually weighed at 1, 4, 7, 10, and 12 d of age, and half of the challenge and control pens were weighed at 14 d of age and the other half at 15 d of age. The BW at 14 or 15 d of age was treated as 1 trait.

At 7 d of age, starting at 0700 h until <1500 h, blood samples (<1 mL) of individual chicks were taken from the wing vein with a 2.0 syringe and 0.5 · 16 mm needle and subsequently transferred to a heparine- (thromboliquine) coated, round-bottomed polystyrene tube. The syringe and needle were coated with heparine immediately before the sampling. At 14 and 15 d of age, 2 blood samples per individual were collected from the jugular vein (euthanization by bleeding) directly into round-bottomed polystyrene tubes either coated with heparine or uncoated.

Levels of T_3 and T_4 were measured in the plasma by RIA, as described by Darras et al. (1992), and expressed in nanograms per milliliter. Intraassay CV were 2.5 and 7.5% for T_3 and T_4, respectively. Antisera as well as T_3 and T_4 standards were obtained from Byk-Belga NV (Brussel, Belgium).

Antibody titers (IgG; *AB*) specific to the *E. coli* O78K80 (506) were determined in the plasma by ELISA, as described by Leitner et al. (1990). The detection antibody used was

RαCh IgG(H+L)/PO. An *E. coli* solution of 5×10^8 concentration was heated in warm water (60°C) for 1 h and subsequently sonicated for 1 min. The extinction was measured at 450 nm and expressed as the *AB* = [critical value - logit(Ext_i)]/slope, where the critical value is the value on the standard curve at the critical cutoff point; logit(Ext_i) = ln[Ext_i/(E_{max} - Ext_i)], and slope is the linear slope of the standard curve.

4.2.5 Data Analysis

An ANOVA was used to test for the effect of treatment [i.e., control or challenge group (Model A) and susceptibility to colibacillosis (Model B) on T_3 and T_4 plasma levels at 7 d of age (T_{3d7} and T_{4d7} , respectively), the change in T_3 and T_4 plasma levels from 7 to 14 d of age (ΔT_3 and ΔT_4, respectively), the *E. coli* specific IgG *AB* at 7 d of age (AB_{d7}), and the change in this titer from 7 to 14 d of age (ΔAB). An ANOVA was also used to test for an interaction between genotype and treatment (Model C) on ΔT_3, ΔT_4, and ΔAB. Based on the mortality, lesions, growth retardation, and feeding inhibition, the susceptibility to colibacillosis was defined as 4 categories with increasing susceptibility: 1) chicks without lesions, 2) chicks with airsacculitis and no systemic lesions, 3) chicks with systemic lesions, and 4) chicks that died. The chicks with systemic lesions and chicks that died showed growth retardation, whereas the other chicks did not (Ask et al., 2006b). The *t*-test was applied to test the effect of each level of susceptibility to colibacillosis and the effect of challenge within genotypes, using Tukey's adjustment (the Tukey-Kramer method) to correct for multiple comparisons. The models were

$$Y_{ijklm} = \mu_{ijklm} + \text{TRIAL}_i + \text{CONTCHAL}_j + \text{SEX}_k + \text{GENOTYPE}_l + \text{DAY1415}_m + e_{ijklm} \quad \text{[A]}$$

where Y_{ijklm} = the individual T_{3d7}, T_{4d7}, ΔT_3, ΔT_4, AB_{d7}, or ΔAB in the *i*th trial (TRIAL_i); the *j*th treatment: control or challenge group (CONTCHAL_j); the *k*th sex (SEX_k); the *l*th genotype (GENOTYPE_l); and the *m*th age at which measurements at 14 or 15 d of age were done (DAY1415_m); μ_{ijklm} = the mean; and e_{ijklm} = the random residual effect.

$$Y_{ijklm} = \mu_{ijklm} + \text{TRIAL}_i + \text{SUSCEPT}_j + \text{SEX}_k + \text{GENOTYPE}_l + \text{DAY1415}_m + e_{ijklm} \quad \text{[B]}$$

where Y_{ijklm} = the individual T_{3d7}, T_{4d7}, ΔT_3, ΔT_4, AB_{d7}, or ΔAB in the *i*th trial (TRIAL_i); the *j*th category of susceptibility (SUSCEPT_j); the *k*th sex (SEX_k); the *l*th genotype (GENOTYPE_l); and the *m*th age at which measurements at 14 or 15 d of age were done (DAY1415_m); μ_{ijklm} = the mean; and e_{ijklm} = the random residual effect.

$$Y_{ijklmn} = \mu_{ijklmn} + \text{TRIAL}_i + \text{CONTCHAL}_j + \text{SEX}_k + \text{GENOTYPE}_l + \text{GENOTYPE} \times \text{CONTCHAL}_m \text{ DAY1415}_n + e_{ijklmn} \quad [C]$$

where Y_{ijklmn}= the individual ΔT_3, ΔT_4, or ΔAB in the ith trial (TRIAL_i; the jth treatment: control or challenge group (CONTCHAL_j; the kth sex (SEX_k, the lth genotype (GENOTYPE_l; the mth interaction between genotype and CONTCHAL (GENOTYPE × CONTCHAL_m); and the nth age at which measurements at 14 or 15 d of age were done (DAY1415_n); μ_{ijklmn} = the mean; and e_{ijklmn} = the random residual effect.

Pearson's correlations, within control and challenge group, were calculated between individual BW at 7, 10, 12, and 14 d of age and ΔT_3 or ΔT_4. In addition, correlations within control and challenge group were calculated between individual ΔAB and ΔT_3 or ΔT_4. The correlations were based on data corrected for trial, sex, genotype, and the age at which measurements at 14 or 15 d of age were done.

4.2.6 Ethics

The experiment was approved by the Animal Ethics Committee (Dierexperimentencommissie, Utrecht University, The Netherlands), and chicks were handled accordingly. The Animal Ethics Committee based its decision on "De Wet op Dierproeven" (1996) and on the "Dierproevenbesluit" (1985; http://www.nca-nl.org/).

4.3 Results

In Table 4.1, T_{3d7} and ΔT_3 in the control and challenge group is given. The T_{3d7} was 57% higher in the challenge group than in the control group ($P < 0.001$), but the increase in T_3 from 7 to 14 d of age was 45% lower ($P = 0.032$) in the challenge group than in the control group. There was no significant effect of susceptibility on neither T_{3d7} ($P \geq 0.584$ for all pairwise comparisons) nor ΔT_3 ($P \geq 0.671$ for all pairwise comparisons), although ΔT_3 was 42% higher in the chicks with airsacculitis than in the chicks without lesions and 97% higher than that of the chicks with systemic lesions.

The T_{4d7} and ΔT_4 in the control and challenge group is also given in Table 4.1. There was no significant difference between control and challenge group for T_{4d7} ($P = 0.283$) or for ΔT_4 ($P = 0.660$). There was also no significant effect of susceptibility on T_{4d7} ($P \geq 0.927$ for all pairwise comparisons) or on ΔT_4 ($P \geq 0.704$ for all pairwise comparisons), although ΔT_4 was 32% higher in the chicks with systemic lesions than in the chicks without lesions and the chicks with airsacculitis.

Table 4.1. The plasma levels of T_3 and T_4 ± SE at 7 d of age (T_{3d7} and T_{4d7}, respectively) in ng/mL and the absolute change ± SE from 7 to 14 d of age (ΔT_3 and ΔT_4, respectively) in the control and challenge group[1] and in the challenge group, depending on susceptibility to colibacillosis. The data were analyzed with an ANOVA, adjusting for trial, sex, genotype, and age at measurement[2]

	T_{3d7}	ΔT_3	T_{4d7}	ΔT_4
Control	0.95 ± 0.06[a]	1.13 ± 0.11[a]	8.53 ± 0.33	5.38 ± 0.56
Challenge, total	1.49 ± 0.05[b]	0.78 ± 0.12[b]	9.02 ± 0.31	5.01 ± 0.62
Susceptibility to colibacillosis:				
No lesions	1.53 ± 0.08[b]	0.82 ± 0.16[ab]	9.18 ± 0.47	4.57 ± 0.79
Airsacculitis only	1.21 ± 0.20[b]	1.16 ± 0.45[ab]	8.14 ± 1.19	4.57 ± 2.29
Systemic lesions	1.47 ± 0.12[b]	0.59 ± 0.23[ab]	8.65 ± 0.68	6.05 ± 1.13
Dead	1.52 ± 0.10[b]	-	8.92 ± 0.58	-

[a,b]Different letters within columns are indicative of significant ($P < 0.05$) differences.

[1]Chicks in the challenge group had been intratracheally inoculated with 0.3 mL of an O78K80 *Escherichia coli* solution at 7 d of age, and chicks in the control group had been inoculated with 0.3 mL of PBS.

[2]The total number of measurements in the control group was n = 298 and 297 for T_{3d7} and T_{4d7} and n = 234 and 240 for ΔT_3 and ΔT_4; in the challenge group, n = 336 for T_{3d7} and T_{4d7} and n = 267 and 271 for ΔT_3 and ΔT_4. The total number of measurements in the group without lesions was n = 142 for T_{3d7} and T_{4d7} and n = 118 and 121 for ΔT_3 and ΔT_4; in the group with airsacculitis only, n = 22 for T_{3d7} and T_{4d7} and n = 14 for ΔT_3 and ΔT_4; in the group with systemic lesions, n = 69 for T_{3d7} and T_{4d7} and n = 58 and 59 for ΔT_3 and ΔT_4; and in the group with dead chicks, n = 96 for T_{3d7} and T_{4d7}.

In Table 4.2, the T_{3d7}, ΔT_3, T_{4d7}, and ΔT_4 in the control and challenge group are given for the 8 genotypes. There was a significant effect of genotype on T_{4d7} ($P < 0.001$) and ΔT_4 ($P = 0.004$), but not on T_{3d7} ($P = 0.126$) or ΔT_3 ($P = 0.321$). There was no significant interaction between genotype and challenge for neither ΔT_3 ($P = 0.690$) nor ΔT_4 ($P = 0.417$). Within genotypes, the only significant difference between control and challenge group was in the Sire2 for ΔT_3 ($P = 0.050$) and in the Sire1 for ΔT_4 ($P = 0.029$).

In Table 4.3, the AB_{d7} and ΔAB in the control and challenge group are given. There was no significant effect of neither challenge ($P = 0.276$) nor susceptibility ($P \geq 0.770$) for all pair- wise comparisons) on AB_{d7}, whereas ΔAB was 41% lower (absolute) in the challenge than in the control group ($P = 0.004$). There was no significant effect of susceptibility ($P \geq 0.955$ for all pairwise comparisons) on ΔAB, although ΔAB in the chicks without lesions was 24% higher (absolute) than in the chicks with airsacculitis only and 26% higher (absolute) than in the chicks with systemic lesions.

In Table 4.4, the AB_{d7} and ΔAB in the control and challenge group are given for the 8 genotypes. There was a significant effect of genotype on both AB_{d7} ($P < 0.001$) and ΔAB ($P < 0.001$), but there was no significant interaction between treatment and genotype on ΔAB ($P = 0.107$). Within genotypes, the only significant difference between control and challenge group in ΔAB was in the DamCross ($P = 0.024$) and the SireCross ($P = 0.004$) correlations within control and challenge group between BW at 7, 10, 12, and 14 d of age and ΔT_3 or ΔT_4 were all small and nonsignificant (ranging from 0.08 ± 0.25 to 0.10 ± 0.15). The correlations within control and challenge group between ΔAB and ΔT_3 or

Table 4.2. The plasma levels of T_3 and T_4 ± SE at 7 d of age (T_{3d7} and T_{4d7}, respectively) in ng/mL and the absolute change ± SE from 7 to 14 d of age (ΔT_3 and ΔT_4, respectively) in the control and challenge group[1] for the 8 genotypes[2]

Genotype	T_{3d7}	ΔT_3			T_{4d7}	ΔT_4		
		Control	Challenge	P-value[3]		Control	Challenge	P-value[3]
SlowGrow (3)	1.19±0.12	1.26±0.34	0.89±0.30	0.432	8.32±0.68[ab]	1.88±1.67	2.71±1.49	0.437
Dam1 (A3)	1.29±0.11	1.32±0.30	1.05±0.33	0.659	8.35±0.63[ab]	4.69±1.51	6.08±1.67	0.496
Dam2 (E4)	1.25±0.11	1.14±0.31	0.39±0.38	0.249	10.89±0.63[a]	2.73±1.55	5.29±1.90	0.395
Dam3 (E5)	1.08±0.10	0.85±0.32	1.11±0.37	0.579	6.56±0.62 [b]	9.02±1.65	10.11±1.87	0.632
Sire1 (A2)	1.04±0.10	1.29±0.28	1.13±0.31	0.625	8.70±0.62[ab]	7.97±1.42	3.39±1.56	0.029
Sire2 (E3)	1.17±0.10	1.14±0.32	0.09±0.34	0.050	10.31±0.63[a]	4.75±1.64	3.38±1.68	0.850
DamCross (A3×E4)	1.27±0.11	1.36±0.33	1.05±0.37	0.378	10.06±0.64[a]	5.59±1.64	5.42±1.86	0.728
SireCross (A2×E3)	1.46±0.11	0.72±0.32	0.47±0.41	0.651	7.01±0.63 [b]	6.04±1.61	4.27±2.07	0.437

[a-b]Different letters within columns are indicative of significant ($P < 0.05$) differences.

[1]Chicks in the challenge group had been intratracheally inoculated with 0.3 mL of an O78K80 *Escherichia coli* solution at 7 d of age, and chicks in the control group had been inoculated with 0.3 mL of PBS.

[2]The data were analyzed with an ANOVA, adjusting for trial, sex, genotype, and age at measurement. The total number of measurements per genotype for T_{3d7} and T_{4d7} ranged from 67 to 84, and the total number of measurements per genotype for ΔT_3 and ΔT_4 ranged from 26 to 37 in the control group and from 28 to 41 in the challenge group.

[3]Significance of within genotype differences in ΔT_3 and ΔT_4 between the control and challenge group.

ΔT_4 were all small and nonsignificant as well (ranging from -0.02 ± 0.82 to -0.08 ± 0.23).

Table 4.3. The *Escherichia coli*-specific IgG antibody titer ± SE at 7 d of age (AB_{d7}) and the absolute change ± SE from 7 to 14 d of age (ΔAB) in the control and challenge group[1] and in the challenge group depending on susceptibility to colibacillosis[2]

	AB_{d7}	ΔAB
Control	4.05 ± 0.16	-1.82 ± 0.17[a]
Challenge, total	3.82 ± 0.15	-1.08 ± 0.19[b]
Susceptibility to colibacillosis:		
No lesions	3.61 ± 0.23	-1.20 ± 0.24[ab]
Airsacculitis only	3.84 ± 0.56	-0.91 ± 0.58[ab]
Systemic lesions	3.71 ± 0.34	-0.89 ± 0.36[ab]
Dead	4.09 ± 0.28	-

[a-b]Different letters within columns are indicative of significant ($P < 0.05$) differences.

[1]Chicks in the challenge group had been intratracheally inoculated with 0.3 mL of an O78K80 *E. coli* solution at 7 d of age, and chicks in the control group had been inoculated with 0.3 mL of a PBS.

[2]The data were analyzed with an ANOVA, adjusting for trial, sex, genotype, and age at measurement. The total number of measurements in the control group was n = 305 for AB_{d7} and 275 for ΔAB; in the challenge group, n = 352 for AB_{d7} and 223 for ΔAB. The total number of measurements in the group without lesions was n = 150 for AB_{d7} and 138 for ΔAB; in the group with airsacculitis only, n = 25 for AB_{d7} and 23 for ΔAB; in the group with systemic lesions, n = 70 for AB_{d7} and 62 for ΔAB; and in the group with dead chicks, n = 103 for AB_{d7}.

Table 4.4. The *Escherichia coli*-specific IgG antibody titer ± SE at 7 d of age (AB_{d7}) and the absolute change ± SE from 7 to 14 d of age (ΔAB) in the control and challenge group[1] for the 8 genotypes[2]

Genotype	AB_{d7}	ΔAB		P^3
		Control	Challenge	
SlowGrow (3)	3.11 ± 0.31[a]	-0.97 ± 0.52[ab]	-0.07 ± 0.45[ab]	0.173
Dam1 (A3)	3.13 ± 0.32[a]	-1.19 ± 0.48[ab]	-0.69 ± 0.51[ab]	0.397
Dam2 (E4)	4.52 ± 0.30[b]	-2.14 ± 0.45[ab]	-1.11 ± 0.55[ab]	0.226
Dam3 (E5)	4.13 ± 0.30[ab]	-1.33 ± 0.44[ab]	-2.26 ± 0.60[ab]	0.524
Sire1 (A2)	3.18 ± 0.30[a]	-0.40 ± 0.44[a]	-0.53 ± 0.51[ab]	0.897
Sire2 (E3)	4.78 ± 0.30[bc]	-3.10 ± 0.50[bc]	-2.15 ± 0.51[b]	0.115
DamCross (A3×E4)	3.00 ± 0.30[a]	-1.18 ± 0.45[ab]	0.32 ± 0.51[c]	0.024
SireCross (A2×E3)	5.63 ± 0.30[c]	-4.49 ± 0.51[c]	-2.31 ± 0.56[b]	0.004

[a-c]Different letters within columns are indicative of significant ($P < 0.05$) differences.

[1]Chicks in the challenge group had been intratracheally inoculated with 0.3 mL of an O78K80 *E. coli* solution at 7 d of age, and chicks in the control group had been inoculated with 0.3 mL of a PBS.

[2]The data were analyzed with an ANOVA, adjusting for trial, sex, genotype, and age at measurement. The total number of measurements per genotype for AB_{d7} ranged from 75 to 86, and the total number of measurements per genotype for ΔAB ranged from 34 to 40 in the control group and from 41 to 46 in the challenge group.

[3]Significance of within line differences in ΔAB between the control and challenge group.

4.4 Discussion

To compare temporal change in plasma T_3 and T_4 and *E. coli*-specific IgG antibody levels in the challenge group relative to the control group, it is of importance that the groups are similar before the challenge with regards to these parameters. In this study,

this was the case for both T_{4d7} and AB_{d7}, as the control and challenge groups did not differ significantly (Tables 4.1 and 4.3). The nonsignificant difference in AB_{d7} between the control and challenge group suggests that there were no differences between the groups in maternal antibodies. In contrast, T_{3d7} was found to be 57% higher in the challenge than in the control group (Table 4.1). This difference is probably related to the differential timing of the blood sampling, as samples were taken from the challenge group from 1 to 5 h after lights on and from 5 to 9 h after lights on from the control group. The eating pattern of broilers is known to be affected by the circadian rhythm; for example, a relatively large peak in feed intake is generally observed within 2 to 3 h after lights on. This peak is associated with an increased T_3 plasma level (Decuypere and Kühn, 1984). Therefore, eating pattern in association with circadian rhythm may explain the observed difference in T_{3d7}.

The ΔT_3 was found to be lower in the challenge group than in the control group, whereas ΔT_4 did not differ significantly between the control and challenge groups (Table 4.1), which suggests functional hypothyroidism in the challenge group relative to the control group. The lower ΔT_3 in the challenge group indicates that metabolic changes have taken place due to the challenge. The groups of differing susceptibility to colibacillosis did not differ significantly for T_{3d7} or T_{4d7} (Table 4.1). This suggests that differences in plasma thyroid hormones before challenge did not influence the susceptibility to the challenge.

Based on this experiment, susceptibility to colibacillosis, as indicated by gross lesions and mortality, was found to be associated with growth retardation (Ask et al., 2006b) and reduced eating behavior, indicating reduced feed intake (Ask et al., 2004). Previously, a positive association has been observed between growth and T_3 in the plasma, and a negative association has been observed between growth and T_4 in the plasma (Lauterio et al., 1986; Decuypere and Buyse, 1988). Reversely, many studies have shown that feed restriction leads to reduced T_3 plasma levels and increased T_4 plasma levels (May, 1978; Darras et al., 1995; Van der Geyten et al., 1999). These associations between growth or feed intake and thyroid hormones could, however, not be confirmed by this study, because the groups of differing susceptibility to colibacillosis did not differ significantly for neither ΔT_3 nor ΔT_4 (Table 4.1). It should be noted, however, that the group with systemic lesions (and growth retardation and absence of eating behavior) did have both the lowest ΔT_3 and the highest ΔT_4 of all the groups.

The ΔAB was found to be lower (in absolute terms) in the challenge group than in the control group (Table 4.3), thereby indicating an elicitation of an antibody response. This can be explained as follows: In the control group, a negativeΔAB was expected due to the degradation of maternal antibodies. In the challenge group, if no antibody response

was elicited, the ΔAB was expected to be either more negative than in the control group (in case of a systemic infection), because of the formation of antibody-antigen complexes or not to differ from the control group (in case of a nonsystemic infection). This study is therefore in agreement with the theory that an immune response (innate or humoral) is accompanied by metabolic changes, as previously observed in, for example, an increased fractional disappearance rate of T_3 in the plasma (Beisel, 1975). There was no significant difference in AB_{d7} among groups of differing susceptibility to colibacillosis, either (Table 4.3). This suggests that the differences in susceptibility to colibacillosis were not related to differences in maternal antibodies. The *E. coli* specific antibody response tended to increase (decreasing ΔAB) with increasing susceptibility (Table 4.3).

The low and nonsignificant correlations between ΔAB and ΔT_3 or ΔT_4 suggested that there was no association between the thyroid hormone changes in the plasma during the challenge and the elicitation of a specific antibody response to the challenge. There was a significant effect of genotype on T_{4d7} , ΔT_4, AB_{d7}, and ΔAB, thereby indicating the presence of genetic variation in these traits, which is in agreement with previous studies (Bowen and Washburn, 1984; Cheng et al., 1991; Yonash et al., 1996). The effect of genotype on T_{3d7} and ΔT_3 was not significant. Although this is not indicative of the presence of genetic variation, it is also not indicative of the absence of genetic variation and therefore not in disagreement with the previous finding of genetic variation in the T_3 plasma level (Bowen and Washburn, 1984). The absence of a significant interaction between genotype and challenge in all traits suggested that genotype does not have an effect on the changes in T_3 and T_4 plasma levels during challenge and also not on the specific antibody response to challenge. Previously, several studies have suggested that the regulation of T_3 and T_4 has been altered in commercial broilers due to the intensive selection for growth traits, although there is controversy on whether this is expressed in the form of functional hypothyroidism (relatively low T_3 but unchanged T_4 levels; May and Marks, 1983; Gonzales et al., 1999) or functional hyperthyroidism (relatively high T_3 but unchanged T_4 levels; Decuypere and Kühn, 1988). Because the SlowGrow was less intensively selected than the other genotypes in this study, it was therefore also expected that the other genotypes would express some degree of functional hypothyroidism or hyperthyroidism relative to the SlowGrow. Neither of the 2 could be confirmed though, as differences between the SlowGrow and the other genotypes were nonsignificant for both ΔT_3 and ΔT_4 (Table 4.2). There were indications of alterations in the T_4 plasma level during challenge due to selection for growth traits, however. The genotypes that have been selected relatively less intensively for growth traits (the SlowGrow and the 3 dam genotypes) tended to respond differently to the *E. coli*

challenge with regards to ΔT_4 than the genotypes that have been selected relatively more intensively for growth traits (the 2 sire genotypes and the SireCross). In the genotypes selected relatively less intensively for growth traits, the ΔT_4 was higher in the challenge than in the control group, whereas the opposite was found in the genotypes selected more intensively for growth traits (Table 4.2).

The ΔAB within genotypes only differed significantly between control and challenge group for the DamCross and the SireCross, but, in general, there was a tendency to a lower (in absolute terms) ΔAB in the challenge than in the control group (except for the Dam3 and Sire1; Table 4.4). As mentioned above, this is indicative of an elicitation of an antibody response. Previously, it has been shown that less intensively selected broilers (broilers randomly selected since 1957) have a higher humoral immune response than more intensively selected broilers (commercial broiler strains from 1991 and 2001; Qureshi and Havenstein, 1994; Cheema et al., 2003), but in this study, the differences in ΔAB between the genotypes that have been selected relatively more or less intensively for growth traits could not support such an effect of selection.

In general, it should further be noted that even though most pairwise comparisons among genotypes were statistically nonsignificant, several were large (e.g., in the challenge group, ΔT_3 was more than 11 times as large in Sire2 as in Sire1), and it cannot be ruled out that such large differences may be of biological significance.

To summarize, there were both an *E. coli*-specific antibody response and thyroid hormone changes in the plasma in response to challenge. There were, however, only indications of an association between susceptibility to colibacillosis and the thyroid hormone changes or the *E. coli*-specific antibody response, and there was no association between maternal antibodies and susceptibility to colibacillosis. The biological background of the differences in susceptibility to colibacillosis is therefore still unclear, but part of the explanation may be found in other metabolism or immune response-related parameters than the ones measured here. There was genetic variation present in both the T_4 plasma level and the *E. coli*-specific antibody response, but genotype did not have a significant effect on ΔT_3 or ΔT_4 during challenge and also not on the specific antibody response to challenge. Further, there were indications of alterations in ΔT_4 during challenge due to the selection for growth traits, as indicated by a lower ΔT_4 in the challenge group relative to the control group for more intensively selected genotypes as opposed to a higher ΔT_4 for less intensively selected genotypes.

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Chapter 5: Modeling the Development of Immunocompetence and Immunoresponsiveness to Challenge in Chicks

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Abstract: The purpose of this study was two-fold: 1) to develop a deterministic model that describes the development of immunocompetence and the kinetics of immunoresponsiveness to a pathogenic challenge in chicks; and 2) to use this model to illustrate the importance of factors in experimental design, such as type of variable measured, measurement timing, and challenge age. Difficulties in evaluating immunological variables hinder attempts to improve animal health through selection on immunological variables. In young chicks, evaluating immunological variables is additionally complicated by immune system development and maternal immunity. The evaluation of immunocompetence and immunoresponsiveness, and the definition of appropriate challenge and measurement strategies, may be enabled through a mathematical model which captures the key components of the immune system and its development. Therefore, a model was developed that describes the development of immunocompetence as well as the kinetics of immunoresponsiveness to a pathogenic extra-cellular bacterial challenge in an individual chick from 0 to 56 days of age. The model consisted of four components describing immunocompetence (maternal- and baseline immunity) and immunoresponsiveness (acute phase- and antibody response). Individual component equations generally fitted published data adequatelly. Four scenarios that represented combinations of challenge age and measurement timing were simulated. In each scenario, the immunoresponsiveness to a particular challenge was compared for three different levels of baseline immunity, representing three broiler genotypes. It was illustrated that experimental design (type of immunoresponsiveness measured, measurement timing, and challenge age) can have an important effect on the ranking of genotypes, groups, or individuals, and on the reliability of extrapolations based on this ranking. It is concluded that this model is a potentially useful tool in the definition of appropriate challenge and measurement strategies when evaluating immunocompetence and immunoresponsiveness. Further, it may be used as a generator of hypotheses on global immunological relationships to be tested experimentally.

Keywords: *Breeding, Chicken, Experimental Design, Immunocompetence, Immunoresponsiveness, Mathematical Modeling*

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5.1 Introduction

Selection for improved immunocompetence (ability to resist and recover from infection and disease; Owens and Wilson, 1999) and immunoresponsiveness (the ability to mount an immune response) has been suggested as a method to improve animal health. However, in selection experiments, expected improvements have not been achieved (Yunis et al., 2002), possibly because selected immunological variables were not indicative of the goal (Knap and Bishop, 2000). For example, different immunological variables are activated depending on whether a challenge is an extra- or intracellular pathogen, and selection is often based on one variable only (Pinard-van der Laan and Monvoisin, 2000; Adkins et al., 2004).

Alternatively, the failures may have been due to complications in the evaluation of individuals relative to other individuals based on any given variable. Complications in these evaluations are introduced by experimental design in combination with immune system development as well as maternal immunity in young animals, such as chicks (Cheema et al., 2003). These complications are due to, for example, challenge timing and the number of measurements over time, but also due to the (relative) timing of these measurements. For example, selection is often based on measurements from one time point only, but measurements from different time points may result in different or contradictory results. Therefore, experimental design in combination with immune system development and maternal immunity may explain why expected improvements in immunocompetence based on selection have not been achieved. Defining appropriate challenge and measurement strategies should, therefore, accommodate successful selection for improved animal health based on immunological variables.

The definition of appropriate challenge and measurement strategies for evaluation of immunocompetence and immunoresponsiveness for selection purposes may be assisted by a mathematical simulation model which describes the development of the immune system. Such a model (currently not available) would allow many scenarios to be tested quickly before doing expensive and time-consuming experiments, and thereby minimize the use of laboratory animals.

The purpose of this study was two-fold: 1) to develop a deterministic model that describes immunocompetence development and immunoresponsiveness kinetics in

chicks challenged with an extra-cellular bacterial pathogen; and 2) to use this model to illustrate the importance of factors in experimental design, such as type of variable measured, measurement timing, and challenge age.

5.2 Materials and Methods

5.2.1 Model Overview

A model was developed that describes the development of immunocompetence as well as the kinetics of immunoresponsiveness to a pathogenic extra-cellular bacterial challenge in an individual chick from 0 to 56 days of age. Immunocompetence was modeled as a combination of two components that are present irrespective of challenge, namely maternal immunity and baseline immunity, defined as the potential ability of the chick to recognize and respond to challenge. Immunoresponsiveness was modeled as a combination of two components that emerge only in response to a challenge, namely acute phase- and antibody response. Each of the components was defined by an equation describing the development or kinetics of that component.

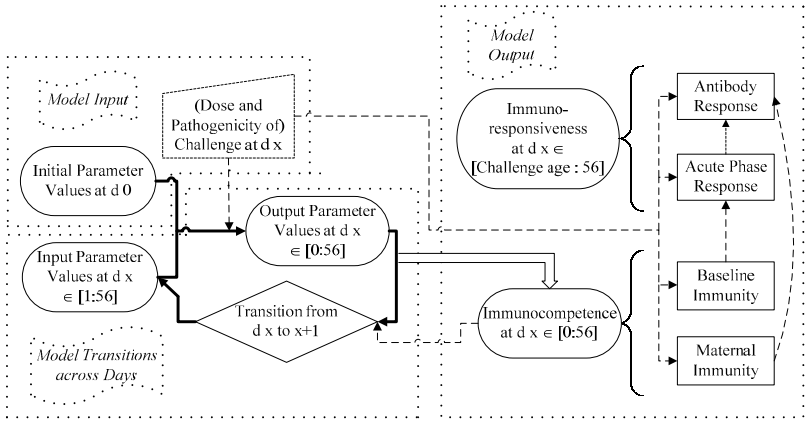

Figure 5.1. An illustration of the overall framework of the model, which describes the development of the immunocompetence in, and the immunoresponsiveness of, an individual chick, depending on challenge, from 0 to 56 d. The three polygonal boxes with dotted lines are indicative of the model input, model transitions across d, and model output respectively. The solid arrows show the transitions of parameter values across d, thus the transitions of the initial level and degradation rate of the maternal immunity, mi_0 and *rmi*, and the initial level, development rate, and expected mature level of the baseline immunity, bi_0, *kbi*, and *cbi*. The open block-arrow shows the transition of output parameter values into the equations defined for immunocompetence. The stippled arrows pointing from box A to B indicate an effect of box A on box B.

The cell-mediated immune response was not modeled, because the model was restricted to extra-cellular pathogens, for which the cell-mediated immune response is of relatively low importance (Janeway et al., 2005). Possible effects of immunological memory were not modeled, because for selection purposes, chicks are normally

challenged once only, and immunoresponsiveness is therefore not affected by immunological memory. For simplicity, spatial effects of infection (e.g. routes of infection) and immune response were not modeled either.

The model input includes initial parameter values at 0 days of age for the immunocompetence equations, challenge age, and challenge pathogenicity. The parameter values at all subsequent ages (1 to 56 days of age) are based on the parameter values at the preceding age and possible effects of challenge. The model output is the immunocompetence and immunoresponsiveness at any particular age. The overall framework of the immunocompetence model is shown in Figure 5.1.

5.2.2 Model Components

Challenge. The challenge was assumed to be an extra-cellular bacterial pathogen. The pathogenicity of a challenge is the disease producing capacity of a pathogen. In this model, the pathogenicity of the challenge was defined as the capacity to cause an immune response (acute phase or antibody response). It was assumed that the pathogenicity could be reflected by the quantity of the pathogen in the host. Pathogen quantity in the host over time can be represented by a sigmoid pattern defined by Gompertz curve, which traditionally describes population growth under control. Given a combination of initial challenge load ($chal_0$), proliferation rate (k_{chal}), and proliferation limit (due to host environment; a_{chal}) of the challenge pathogen, the pathogen quantity, i.e. pathogenicity of the challenge, becomes:

$$[1] \qquad Chal(age) = chal_0 + a_{chal}.\exp\left[-\exp[-i.k_{chal}.(age - age_{chal})]\right],$$

where i is a scaling parameter to make $Chal(age_{inf}) \approx chal_0$ (see Table 5.1); age is the age in days; and age_{chal} is the age at challenge in days. The lower and upper limits of possible values of $chal_0$, k_{chal}, and a_{chal} are given in Table 5.2. The challenge was assumed to be given once in a chicks lifetime (0 to 56 days of age) at a certain age, age_{chal}, and re-challenge was not modeled.

Maternal Immunity. Maternal immunity consists of antibodies transferred from the dam through the yolk. The level of maternal antibodies increases during the first 2-4 days of age (as the yolk is absorbed) (Kaleta et al., 1977; Kowalczyk et al., 1985; Shawky et al., 1994), whereafter it decreases until 2 to 4 weeks of age (Smith et al., 1994; Jeurissen et al., 2000; Ahmed and Akhter, 2003). The antibodies are degraded at an increasing rate over time in accordance with age related metabolic changes (Stormont, 1972; Kaleta et al., 1977). Mathematically, the degradation can be described as exponential and expressed with a half-life constant (Kaleta et al., 1977; Sarvas et al., 1993; Sahin et al., 2001; Wilson et al., 2001; Müller et al., 2002, 2005; Tizard,

2002). Therefore, the kinetics of the maternal immunity, *MI*, were described with the following equation (2), which results in an exponential decrease of maternal immunity with increasing age, tending towards zero (Figure 5.2):

[2] $$MI(age) = mi_0 . \exp[- rmi(age).age],$$

where mi_0 is the initial level and *rmi* is the degradation rate. The lower and upper limits of mi_0 and *rmi* are given in Table 5.2. The equation of *MI* (2) approaches the zero asymptote very slowly, which is not in agreement with the biological expectation that maternal antibodies have disappeared by 2 to 4 weeks of age. Therefore, it was assumed that $MI = 0$ for $MI < 0.001$.

Table 5.1. The values of the scaling parameters for the immunoresponsiveness components, the acute phase and antibody response, as well as for the effect of challenge on the maternal immunity

Scaling parameter[1]	Value
c	6.22
s_1	2.00
s_2	0.10
s_3	1.00
s_4	0.33
s_5	4.00

[1] c is a scaling parameter for the challenge; s1 and s2 are scaling parameter for the acute phase response; s3 and s4 are scaling parameters for the antibody response; and s5 is a scaling parameter for the factor by which the parameter rmi increases (F_{rmi}) at the age of challenge.

Table 5.2. The lower and upper limits of parameter values for the immunocompetence components, maternal immunity and baseline immunity, and the challenge

Parameter	Lower limit	Upper limit
$chal_0$[1]	0.100	1.0
kchal[1]	0.100	1.5
achal[1]	0.100	1.0
mi_0[2]	0.000	1.0
rmi[2]	0.001	-
bi_0[3]	0.010	1.0
kbi[3]	0.001	-
abi[3]	0.010	1.0
cbi[3]	-0.600	1.0

[1] $chal_0$, k_{chal}, and a_{chal} are the initial challenge load, proliferation rate of the challenge pathogen, and proliferation limit of the challenge pathogen respectively.

[2] mi_0 and *rmi* are the initial maternal immunity and the degradation rate of maternal immunity respectively.

[3] bi_0, *kbi*, and *abi* are the initial baseline immunity, the development rate, and the asymptotic baseline immunity respectively, and *cbi* is a parameter, which in combination with bi_0 prescribes the asymptotic baseline immunity.

Baseline Immunity. Baseline immunity is assumed to represent the potential ability of the chick to recognize and respond to challenge. It is assumed to consist of the basal levels of the innate immunity, natural antibodies, and lymphocytes. The innate immunity includes anatomical, chemical, and physiological barriers, cellular elements (endocytic, phagocytic, and antigen presenting cells, e.g. macrophages and dendritic cells), and soluble factors such as the complement system.

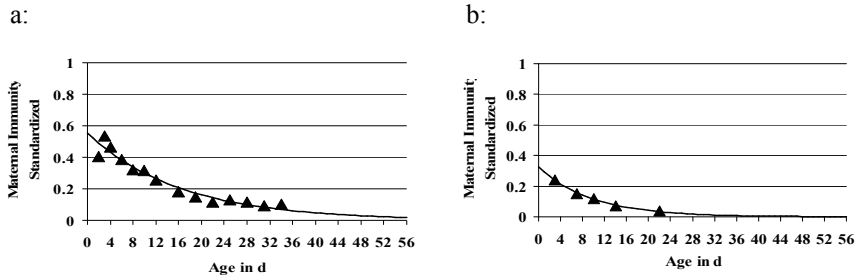

Figure 5.2. Model prediction versus published data *(▲, data; ‾, model)* for the degradation of maternal immunity illustrated by data a; $R^2 = 0.94$ (Kaleta, 1972), and data b; $R^2 = 0.99$ (Islam et al., 2002). Kaleta (1972) measured Newcastle Disease Virus specific maternal antibodies in 10 chicks by means of a so-called virus-neutralization-test and expressed as so-called neutralization-indices. Islam et al. (2002) measured maternal antibodies, expressed as the absorbance, in the plasma of healthy broiler chicks by means of an ELISA.

The pattern of development of baseline immunity should reflect the combined effect of all the above-mentioned elements and reflect immunocompetence, excluding maternal immunity. The immunocompetence of young and immature vertebrates, including chicken, has been described as gradually increasing with age until maturity, where a given plateau is reached (Siegrist, 2001; Reese et al., 2006). This has, for example, been demonstrated by age-dependent antibody responsiveness (Nadler et al., 1980; Siegrist, 2001), where actual antibody responses are assumed to reflect the ability to mount an immune response. This may be related to, amongst others, a reduced capacity of dendritic cells to communicate with lymphocytes (Marshall-Clarke et al., 2000; Morein et al., 2002). A gradual increase with age is also seen for different elements of innate immunity. For example, age-dependent deficiencies have been demonstrated in macrophage and heterophil phagocytosis and killing (Kodama et al., 1976; Wells et al., 1998), as well as for natural antibodies (Kramer and Cebra, 1995; Parker et al., 1996; Parmentier et al., 2004) and the quantity and functionality of lymphocytes (Anderson and Stephens, 1970; Klinman, 1976; Van Benten et al., 2005).

The development of the baseline immunity with age has not previously been modeled. Based on the above-mentioned observations the kinetics of baseline immunity, *BI*, were described with the following equation (3), which results in a curvilinear increase from some given initial level towards some given asymptotic mature level (Figure 5.3) . This equation allows for relatively large flexibility in the development pattern of baseline immunity, enabling it to describe patterns differing among individuals as well as patterns differing among different underlying immunological variables. For example, the equation allows for an almost flat development to an exponential increase until the mature plateau is reached.

[3] $$BI(age) = 1/\left[\left[1/\left[bi_0 - cbi(age)\right]\right] - 1\right] + \exp\left[-kbi(age).age\right]\right] + cbi(age),$$

where bi_0 is the initial level; *kbi* is the rate of increase; and *cbi* is a constant, which in combination with bi_0 prescribes the asymptote:

[4] $$BI_{asymptote} = 1/\left[1/\left[bi_0 - cbi(age)\right]\right] - 1\right] + cbi(age).$$

The lower and upper limits of bi_0, *kbi*, and *cbi* are given in Table 5.2.

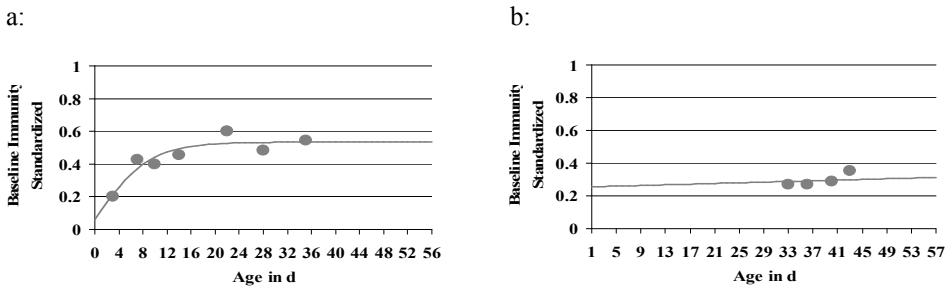

Figure 5.3. Model prediction versus published data *(●, data; ‒, model)* for the development of baseline immunity illustrated by data a; $R^2 = 0.85$ (Islam et al., 2002), and data b; $R^2 = 0.77$ (Okamura et al., 2004). Islam et al. (2002) measured total CD45+/CD3+ lymphocyte (T-cells) count in the plasma of healthy broiler chicks. Okamura et al. (2004) measured serum IFN-γ in healthy chickens.

Acute Phase Response. The acute phase response consists of innate immune responses to antigenic stimulation including releases of cytokines, acute phase proteins, complement system factors, fever, phagocytic and cytotoxic activity, and direct cell killing. The onset of the acute phase response occurs almost immediately after infection, and it normally lasts for a few days only (Stvrtinova et al., 1995). The response pattern is relatively similar, regardless of the pathogen or infection route, though differences may be observed in quantity and temporal characteristics (Nair, 1973). It is usually bell-shaped, but sometimes skewed to either side (Hallquist and Klasing, 1994; Xie et al. 2002; Juul-Madsen et al., 2003; Kaiser et al., 2003), and has also previously been modeled as such (Antia and Koella, 1994; Faro et al., 1997; Morel, 1998). The acute phase response, *APR*, kinetics were therefore described by the following equation (5), resulting in an inverse parabolic pattern (Figure 5.4):

[5] $$APR(age - age_{APR}) = s_1\left[-APR_a\left[s_2.(age - age_{APR})^2\right] + APR_b\left[s_2.(age - age_{APR})\right]\right],$$

where age_{APR} is the age at which the acute phase response is initiated relative to the age at challenge; APR_a and APR_b prescribe the dependence of the magnitude of the acute phase response on challenge and baseline immunity, as described in Section '*Interrelations among Model Components*' below (equations 11 and 12); and s_1 and s_2 are scaling parameters (Table 5.1).

The acute phase response was assumed to be initiated at a certain age after challenge, age_{APR}, with the time lag between challenge and initiation of the acute phase response decreasing with increasing baseline immunity at the age of challenge. The age_{APR} was defined by the following equation (6), resulting in an interval of 0 to 10 days post challenge:

[6] $$age_{APR} = age_{chal} + 10\left[1 - BI(age_{chal})^{0.5}\right]$$

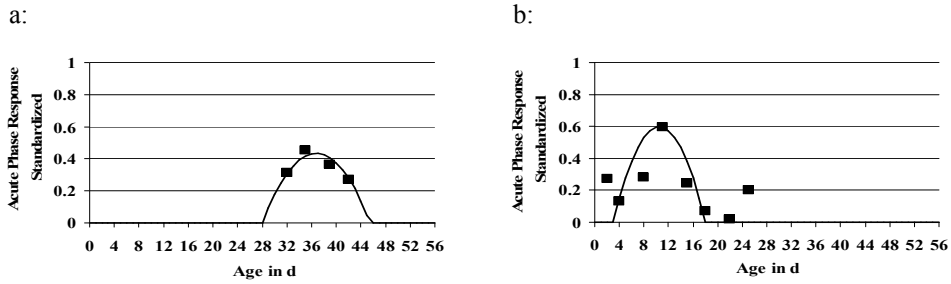

Figure 5.4. Model prediction versus published data *(■, data; ━, model)* for the kinetics of the acute phase response illustrated by data a; $R^2 = 0.82$ (Okamura et al., 2004), and data b; $R^2 = 0.57$ (Jarosinski et al., 2002). Okamura et al. (2004) measured serum IFN-γ in chickens vaccinated with a killed salmonella at 4 wk. The model predictions were based on the assumption of a challenge at 4 wk. Jarosinski et al. (2002) measured plasma nitric oxid levels (as indicated by its by-product nitrite) in experimental SPF chicken lines challenged with Marek's disease virus at 1 d. The model prediction was also based on a challenge at 1 d.

Antibody Response. The antibody response is a part of the acquired immune system, which is produced by B-cells and mediated by Th2-cells in response to antigenic stimulation (Janeway et al., 2005). The antibody response in young chicks is immature, but a large range of responsiveness has been observed. Chicks generally respond faster and more strongly with increasing age (Hatkin et al., 1993), which is probably, amongst other factors, related to the development of the immune system (O'Neill et al., 2006). Antibody response kinetics can principally be described as having four phases, comprising a latent phase, a phase of exponential increase, a steady-state phase, and a reduction phase (Benjamini and Leskowitz, 1991). The latent phase varies between 0 and 3 days (Gross and Siegel, 1975; Leitner et al., 1992), and the peak is reached between 5 and 15 days post challenge (Ubosi et al., 1985; Kreukniet and van der Zijpp, 1990; Leitner et al., 1992). The kinetics of antibody responses depend on the immunoglobulin Isotype. A primary antibody response consists of mainly IgM rather than IgG (Glick, 1995; Tizard, 2002), and in the initial phase of a secondary response IgM prevails as well. The IgM response is relatively short-lived and decreases more rapidly, relative to the IgG response (Bacon et al., 1972). In infants, IgM is the predominant Isotype in antibody responses, and therefore the kinetics of the antibody

response, *ABR*, were described with the following gamma equation (7), which results in a sigmoid exponential pattern towards peak production, after which it gradually decreases towards zero (Figure 5.5). The absence of a true steady-state phase was assumed to be realistic because of the young age of the chicks.

[7] $$ABR(age - age_{ABR}) = ABR_a.(age - age_{ABR})^{s_3} \exp[-s_4.(age - age_{ABR})],$$

where age_{ABR} is the age at which the antibody response is initiated; ABR_a describes the dependence of the antibody response on the challenge, maternal immunity, and acute phase response, as described in Section '*Interrelations among Model Components*' below (equation 15); and s_3 and s_4 are scaling parameters (Table 5.1).

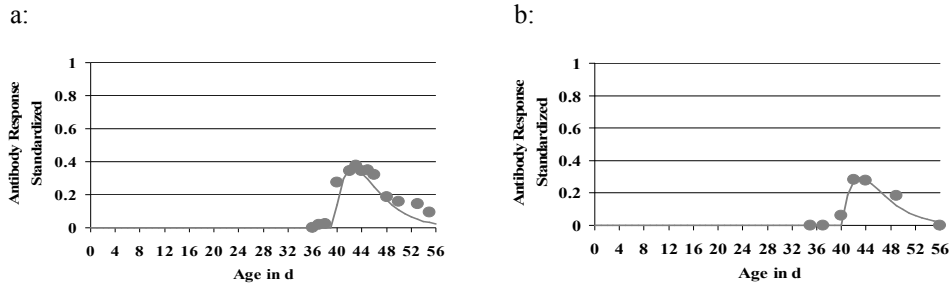

Figure 5.5. Model prediction versus published data *(●, data; ⁻, model)* for the kinetics of the antibody response illustrated by data a; $R^2 = 0.94$ (Parmentier et al., 1998), and data b; $R^2 = 0.91$ (Ubosi et al., 1985). Parmentier et al. (1998) measured the total antibody response in the serum to a combination of the two antigens SRBC and BSA in heavy, brown layer chicks inoculated at 35 d by means of an ELISA, and expressed it as the $\log_2$ of the greatest dilution giving a positive reaction. Ubosi et al. (1985) measured the antibody response to SRBC in chicks of the type White Leghorn inoculated at 35 d by means of the microtiter procedure, and expressed it as the $\log_2$ of the reciprocal of the greatest dilution giving visible agglutinin. The model predictions were based on a challenge at 35 d in both data a and b.

The antibody response was assumed to be initiated at a certain age after the challenge, age_{ABR}, with the time lag between the challenge and initiation of the antibody response decreasing with a decreasing time lag between the challenge and initiation of the acute phase response. The age_{ABR} was defined by the following equation (8):

[8] $$age_{ABR} = age_{APR} + 10\ln[s_2.(age_{APR} - age_{chal}) + 1]$$

Interrelations among Model Components. The development of the immunocompetence, the challenge dynamics, and the kinetics of the immunoresponsiveness are interrelated. These interrelations taken into account in this model are illustrated in Figure 5.1 as indicated by the dotted arrows.

The maternal immunity is affected by the challenge, as the encountering of pathogenic antigens results in an increased degradation rate of maternal antibodies (Kaleta et al., 1977; Siegrist, 2001). It was assumed that neither the baseline immunity, nor the acute

phase- or the antibody response had an effect on the maternal immunity, because the maternal antibody is simply degraded in the same way that any other protein is degraded in the bodily fluids (Tizard, 2002). The increased degradation rate of the maternal immunity, *rmi*, in the face of a challenge was described by an increase in the parameter *rmi* by multiplication of *rmi* with a factor, F_{rmi}, at the age of challenge. The factor, F_{rmi}, increases with increasing initial challenge dose and was defined as:

[9] $$F_{rmi} = s_5.chal_0^{0.5},$$

where s_5 is a scaling factor (Table 5.1). This factor reflects an increasing likelihood of the maternal antibodies encountering the antigens with increasing challenge dose. The likelihood of the maternal antibodies encountering antigens is likely to be a function of antigen concentration (Kulin et al., 2002), because of the distribution of antibodies and antigens in the bodily fluids.

There are indications that maternal antibodies can have a suppressing effect on the development of baseline immunity in the form of natural antibodies (Kramer and Cebra, 1995), and there are indications that maternal antibodies positively affect the development of lymphocytes (Lemke et al., 2000). However, no indications have been found that maternal antibodies affect the development of the innate immunity. Because of the opposing effect of maternal antibodies on natural antibodies and lymphocytes and the absence of an effect on innate immunity, for simplicity it was assumed that maternal immunity did not affect baseline immunity. Pathogenic antigenic stimulation of the baseline immunity may result in an increased rate of development and an increased expected mature level. For example, the quantity of natural antibodies increases in response to pathogenic challenge, and the ability to respond to a secondary challenge increases, reflecting acquired memory. Moreover, the maturity of the immune system is increased in response to challenge (Tomer and Shoenfeld, 1988; Mast and Goddeeris, 1999). The increased development rate and mature level of baseline immunity in the face of a challenge was described by an increase in the parameters *kbi* and *cbi* by multiplication of both *kbi* and *cbi* with a factor, F_{BI}, at the age at which the acute phase response reaches its peak, age_{APRmax}.

The effect of challenge on the baseline immunity is reflected in the elicitation of an acute phase response (Section '*Acute Phase Response*' above), and therefore, F_{BI} is defined as a function of the acute phase response as:

[10] $$F_{BI} = 1 + APR_{max}^{0.5},$$

where APR_{max} is the peak of the acute phase response (equation 14). This reflects an increasing effect of the antigenic stimulation of the baseline immunity on its own development.

The acute phase response was assumed not to be affected by the maternal immunity. This was assumed, because no clear demonstration of such an effect has been seen in chicks, and further, acute phase response is likely to be initiated before pathogens come in contact with maternal antibodies, which to our knowledge are mainly present in the blood. In contrast, the acute phase response was assumed to depend on the baseline immunity, because the acute phase response is defined as consisting of innate immune responses (Stvrtinova et al., 1995). The acute phase response was also assumed to depend on the challenge, because the acute phase response is defined as a response to antigenic stimulation. The magnitude of the acute phase response increases with increasing baseline immunity and increasing challenge load (Jacobsen et al., 2004). It was assumed that the effect of the baseline immunity and challenge on the magnitude of the acute phase response could be captured in the parameters APR_a and APR_b as follows:

[11] $$APR_a = chal_0.BI(age_{chal}) \text{ and}$$

[12] $$APR_b = [1 + kchal]chal_0.BI(age_{chal}).$$

These parameter definitions reflect an assumption that the acute phase response is a linear function of the baseline immunity, based on the definition of baseline immunity being the ability to mount an immune response, and the relationship is such that the acute phase response is a function of the challenge given by the initial challenge dose and the proliferation rate of the pathogen. It was assumed that in addition to the initial challenge dose, the proliferation rate of the pathogen would also have an effect on the acute phase response, because of the expected lag between challenge age and age at initiation of the acute phase response.

Entering APR_a and APR_b into the equation for the acute phase response (Section '*Acute Phase Response*' above) and solving $APR'(age\text{-}age_{APR}) = 0$ for *age* gives the age at which the acute phase response reaches its peak:

[13] $$age_{APRmax} = 0.5.[1 + kchal] + age_{APR}.$$

Therefore, it follows that the peak of the acute phase response, APR_{max}, is:

[14] $$APR_{max} = 0.25.\left[s_1.chal_0.BI(age_{chal}).[1 + kchal]^2\right].$$

The antibody response was assumed to be negatively affected by the maternal immunity, because antibody responses have been shown to be inhibited by the presence of maternal antibodies (Siegrist, 2001). It was assumed that any positive effects of maternal antibodies on the antibody response repertoire after fading of the maternal antibodies (Lemke et al., 2000) were not of importance. It was also assumed that the antibody response was affected by the acute phase response, and thereby the baseline immunity, because stimulation by various elements of the innate immunity (response) are necessary for the initiation of the antibody response (Siegrist, 2001). The antibody

response was also assumed to be affected by challenge, because of the definition of the antibody response as a response to antigenic stimulation. The magnitude of the antibody response increases with an increasing acute phase response and challenge load (Janeway et al., 2005), but decreases with an increasing maternal immunity (Siegrist, 2001). It was assumed that the effects of the acute phase response, challenge, and maternal immunity on the magnitude of the antibody response could be captured in the parameter ABR_a as follows:

[15] $$ABR_a = \exp[chal_0.achal].\exp\left[-MI(age_{ABR})^{0.5}\right].\ln[1+APR_{max}] \cdot$$

This parameter definition results in an increasing stimulating effect of the challenge, given by the initial challenge dose and the expected proliferation limit of the pathogen, on the antibody response. It was assumed that not only the initial challenge dose would have an effect on the antibody response, but also the expected proliferation limit of the pathogen, because the duration of an antibody response is dependent on the presence of antigens, and a greater proliferation limit is reflective of the necessity for an increased antibody response.

Maternal immunity inhibits antibody response, and the effect is presumably non-linear. This explanation for this is that at high levels of maternal immunity, the inhibitory effect of a unit increase in maternal immunity on the antibody response is less than at lower levels of maternal immunity. This is based on the assumption that at high levels of maternal immunity, the antibody response is already so strongly inhibited that further inhibition is without effect. Such a non-linear inhibition of the antibody response by the maternal immunity can be reflected by raising the maternal immunity to the power 0.5.

Entering this into the equation for the antibody response (Section '*Antibody Response*' above) and solving $ABR'(age\text{-}age_{ABR}) = 0$ for *age* gives the age at which the antibody response reaches its peak:

[16] $$age_{ABRmax} = s_3/s_4 + age_{ABR}.$$

Therefore, it follows that the peak of the antibody response is:

[17] $$ABR_{max} = ABR_a.[s_3/s_4]^{s_3}.\exp[-s_3].$$

5.2.3 Estimation of Model Parameters

To parameterize the model, the model component equations were fitted to published experimental data. Experimental data were mainly found in the poultry research. However, because of the limited availability of data with sufficient measurements, published studies on mammalian species were also used, as temporal patterns of many

immunological variables in mammalian species appear to be similar to those in poultry (Sharma, 1991).

The parameters of the maternal- and baseline immunity equations (mi_0, *rmi*, bi_0, *kbi*, and *cbi*) were estimated from published experimental data (Table 5.3). When the experimental data on maternal antibodies included more than one measurement from 0 to 4 days of age, then the initial maternal immunity, mi_0, was assumed to be equal to the measurement that was closest to 0 days of age. This was assumed realistic considering the uptake of maternal antibodies from the yolk during this age interval (Kaleta et al., 1977; Kowalczyk et al., 1985; Shawky et al., 1994). The maternal immunity degradation rate, *rmi*, could then be estimated by linear regression. Otherwise, mi_0 and *rmi* were simultaneously estimated by non-linear regression. The parameters of the baseline immunity equation (bi_0, *kbi*, and *cbi*) were estimated from published experimental data by non-linear regression.

The model requires the determination of the values of five parameters. Estimating the values of all of these parameters from experimental data of a single study was difficult because of the data sparsity. Whenever possible, all the parameter values were estimated from the same study, but where sufficient experimental data were not available estimates were suggested based on other studies. For example, in most published studies with experimental data on either acute phase- or antibody response, no data was available on maternal- or baseline immunity, and suggestions for the parameter values therefore had to be made based on other studies.

The published data were standardized into values with restricted intervals (Table 5.2). Standardization of the data was necessary to enable interpretation and comparisons of different immunological variables, which were measured on different scales (e.g. macrophage count and potential nitrite production by macrophages) or by different methods (e.g. ELISA or hemagglutination assay). A linear transformation was used to standardize all values to the interval [0:1]: $z = [x - min]/[max - min]$, where z is the transformed value; x is the published data point; *min* is the minimum; and *max* is the maximum. The minima were set to 0 where this made sense biologically (for example, the titer of maternal antibodies will always decrease to 0 at some point). In some cases it did not make biological sense to set the minima to 0 (for example, leukocyte- or lymphocyte counts), and in those cases the minima were set to the lowest observed value for the particular immunological measure in published studies. Maxima were set to the greatest observed value for the particular immunological measure in published studies using immature animals. The transformation is linear, and the effect of the

Table 5.3. Parameter values (mi_0, rmi, bi_0, kbi, and cbi[1]) either estimated from published experimental data or suggested based on estimates from other studies, where indicated by an octothorpe, #. The age at challenge, age_{chal}[2] was given in each of the studies and the estimates of the challenge parameter values ($chal_0$, k_{chal}, and a_{chal}[3]) were suggested based on the reported dose and pathogenicity of the challenge pathogen used in each of the studies

Published data source	mi_0	rmi	bi_0	kbi	cbi	age_{chal}	$chal_0$	k_{chal}	a_{chal}
Kaleta (1972)	0.55	0.06	0.06	0.22	-0.43	-	-	-	-
Islam et al. (2002)	0.33	0.10	0.06	0.22	-0.43	-	-	-	-
Cawthraw et al. (1994)	0.83	0.15	0.01	0.02	-0.70	-	-	-	-
Dusbábek et al. (1994)	0.83	0.15	0.70	0.01	0.21	-	-	-	-
Okamura et al. (2004)	0.83	0.15	0.26	0.01	-0.24	28	1.00	0.75	1.00
Jarosinski et al. (2002)[4]	0.83	0.15	0.70	0.01	0.21	3	0.75	0.50	0.50
Glass et al. (2003)[4]	0.83	0.15	0.70	0.01	0.21	9	1.00	0.60	1.00
Glass et al. (2005)[4]	0.83	0.15	0.70	0.01	0.21	5	0.80	0.60	1.00
Parmentier et al. (1994)[4]	0.83	0.15	0.10	0.25	-0.35	35	0.30	1.00	0.20
Ubosi et al. (1985)[4]	0.83	0.15	0.10	0.25	-0.35	35	0.40	0.80	0.50

[1] mi_0 and rmi are the initial maternal immunity and the degradation rate of maternal immunity respectively; and bi_0 and kbi are the initial baseline immunity and the development rate of baseline immunity respectively, and cbi is a parameter, which in combination with bi_0 prescribes the asymptotic baseline immunity.
[2] age_{chal} is the age at challenge in d.
[3] $chal_0$, k_{chal}, and a_{chal} are the initial challenge load, proliferation rate of the challenge pathogen, and asymptotic challenge load respectively.
[4] These publications provided data for the acute phase or antibody response or both, but not for the maternal and baseline immunity, and it was therefore necessary to assume parameter values based on other published studies to fit the acute phase and antibody response equations.

choices of minima and maxima is therefore a pure scaling effect, which will not have any effect on the relative outputs of the model (i.e. when comparing individual chicks or group means).

5.2.4 Experimental Design and Evaluation of Immunoresponsiveness

Many factors may affect traits describing immunocompetence and immunoresponsiveness and, hence, the conclusions drawn from experimental studies. These include age at challenge, type of variable measured, and the number and relative timing of measurements. This is of great importance in, for example, comparative studies and genetic evaluations. To explore this, four scenarios that represented combinations of challenge age and measurement timing were simulated. In each scenario, the immunoresponsiveness (acute phase- and/or antibody response) to a particular challenge was compared for three different levels of baseline immunity. The three levels of baseline immunity were illustrative and could, for example, be representative of three broiler genotypes in a line comparison experiment, three family groups, or simply three individual chicks in a genetic evaluation. In the remainder of this paper, the three levels of baseline immunity will be assumed to represent different broiler genotypes. The following scenarios and simulated broiler genotypes were compared:

- $Chal_{14}T_7$ and $Chal_{14}T_{14}$: Challenge at 14 days of age; measurements at 7 and 14 days post challenge respectively.
- $Chal_{35}T_7$ and $Chal_{35}T_{14}$: Challenge at 35 days of age; measurements at 7 or 14 days post challenge respectively.
- L_iH_r: low initial baseline immunity, bi_0, but a high rate of development, *kbi* (bi_0 = 0.10; *kbi* = 0.05; and *cbi* = -0.50; where *cbi* is a parameter, which in combination with bi_0 prescribes the asymptotic baseline immunity).
- H_iL_r: high initial baseline immunity, bi_0, but a low rate of development, *kbi* (bi_0 = 0.50; *kbi* = 0.01; and *cbi* = 0.00).
- H_iH_r: high initial baseline immunity, bi_0, and a high rate of development, *kbi* (bi_0 = 0.50; *kbi* = 0.05; and *cbi* = -0.50).

5.3 Results

5.3.1 Model Fitting

In Figure 5.2, the fit of the modeled maternal immunity is illustrated along with the standardized published data points (Kaleta, 1972; Islam et al., 2002). In both cases, the model equation fitted published data well as measured by the R^2. The model fit to maternal immunity published in other studies was also investigated (Kaleta et al., 1977;

Cawthraw et al., 1994; Shawky et al., 1994; Smith et al., 1994; Boa-Amponsem et al., 1997; Toro et al., 1997; Jeurissen et al., 2000; Mondal and Naqi, 2001; Sahin et al., 2001), though not illustrated here, and the model equation generally fitted this data well too ($0.62 \leq R^2 \leq 1.00$; mean $R^2 = 0.91$ based on 29 cases).

In Figure 5.3, the fit of the modeled baseline immunity is illustrated along with the standardized published data points (Islam et al., 2002; Okamura et al., 2004). In general, the model equation fitted the data well as measured by the R^2. The model fit to measures of baseline immunity published in other studies was also investigated (Burton and Harrison, 1969; Anderson and Stephens, 1970; Cawthraw et al., 1994; Dusbábek et al., 1994; Toro et al., 1997; Jeurissen et al., 2000; Qureshi et al., 2000; Bar-Shira et al., 2003), although not illustrated here, and the model equation generally fitted this data well too ($0.32 \leq R^2 \leq 1.00$; mean $R^2 = 0.87$ based on 47 cases).

In Figure 5.4, the fit of the modeled acute phase response is illustrated along with the standardized published data points (Jarosinski et al., 2002; Okamura et al., 2004). The model generally fitted the data well as measured by the R^2. The model fit to acute phase responses published in other studies was also investigated (Qureshi et al., 2000; Kaiser et al., 2003; Glass et al., 2003, 2005; Withanage et al., 2005), though not illustrated here. The goodness-of-fit to the data from these studies varied considerably, because it attempted to describe the kinetics of a large range of immunological variables by means of one single equation, and these variables do not all show the modeled pattern consistently ($0.001 \leq R^2 \leq 0.98$; mean $R^2 = 0.69$ based on 37 cases). However, the model generally fitted the published data on acute phase proteins and fever well.

In Figure 5.5, the fit of the modeled antibody response is illustrated along with the standardized published data points (Ubosi et al., 1985; Parmentier et al., 1998). Again, the model fitted the data well as measured by the R^2. The model fit to antibody responses published in other studies was also investigated (Kreukniet and van der Zijpp, 1990; Leitner et al., 1990; Cook et al., 1992; Smith et al., 1994), although not illustrated here. The goodness-of-fit to the data from these studies varied, but was good in many cases ($0.31 \leq R^2 \leq 0.98$; mean $R^2 = 0.77$ based on 58 cases). The lower fit was mainly observed for studies where total antibodies or IgG was measured rather than IgM.

5.3.2 Model Output: Experimental Design and Evaluation of Immunoresponsiveness

The immunoresponsiveness of the three broiler genotypes is illustrated in Figure 5.6. The scenarios $Chal_{14}T_7$ and $Chal_{14}T_{14}$ are illustrated in Figure 5.6a and c, and the scenarios $Chal_{35}T_7$ and $Chal_{35}T_{14}$ are illustrated in Figure 5.6b and d.

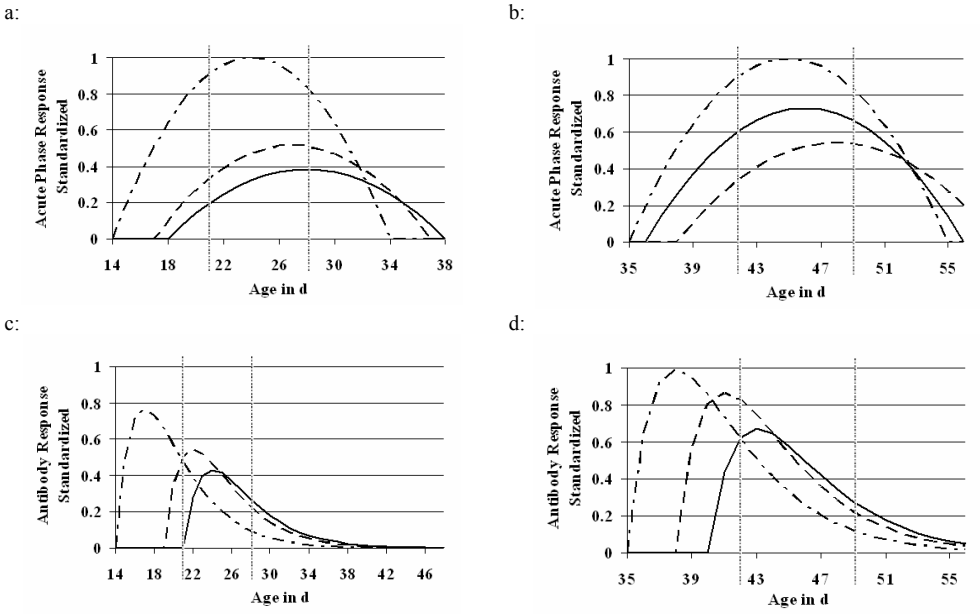

Figure 5.6. Immunocompetence reflected by the acute phase and antibody response in three hypothetical broiler genotypes: L_iH_r (——), H_iL_r (– – –), and H_iH_r (–·–·–). The figures are, a and c: $Chal_{14}T_7$ and $Chal_{14}T_{14}$: challenge at 14 d and measuring of the acute phase (a) and antibody response (c) at 7 or 14 d post challenge, respectively (indicated by the dotted vertical lines); b and d: $Chal_{35}T_7$ and $Chal_{35}T_{14}$: challenge at 35 d and measuring of the acute phase (b) and antibody response (d) at 7 or 14 d post challenge, respectively.

In scenario $Chal_{14}T_7$, H_iH_r is the most immunoresponsive genotype, L_iH_r is the least immunoresponsive genotype, and H_iL_r is intermediate when the evaluation is based on the acute phase response (Figure 5.6a), whereas when the evaluation is based on the antibody response, H_iH_r is the most immunoresponsive genotype along with H_iL_r, and L_iH_r is the least immunoresponsive genotype (Figure 5.6c). Similarly, when the evaluation is based on the acute phase response in scenario $Chal_{35}T_7$, H_iH_r is the most immunoresponsive genotype, H_iL_r is the least immunoresponsive genotype, and L_iH_r is intermediate (Figure 5.6b), whereas when the evaluation is based on the antibody response, L_iH_r is the most immunoresponsive genotype, and H_iH_r is the least immunoresponsive genotype along with H_iL_r (Figure 5.6d). This illustrates the potential influence of the type of immunoresponsiveness measured. Note that, for the acute phase response, the ranking of the two less immunoresponsive genotypes is changed with age of challenge.

Scenario $Chal_{14}T_{14}$, leads to the same conclusion as $Chal_{14}T_7$ with regard to the acute phase response (Figure 5.6a). However, for the antibody response (Figure 5.6c) the ranking of genotypes changes: L_iH_r now appears to be the most immunoresponsive genotype, whereas H_iH_r and H_iL_r are both less immunoresponsive. Again, this illustrates

the potential influence of the type of immunoresponsiveness measured, and moreover it illustrates how the effect of the type of immunoresponsiveness measured interacts with measurement timing. Scenario $Chal_{35}T_{14}$ also leads to the same conclusion as $Chal_{35}T_7$ with regard to the acute phase response (Figure 5.6b), but for the antibody response (Figure 5.6d) H_iL_r is the most immunoresponsive genotype, and H_iH_r is the least immunoresponsive genotype.

To summarize, the potential influence of measurement timing on the evaluation of immunoresponsiveness is illustrated by the differences in the genotype ranking with regard to the acute phase response (Figure 5.6c) between scenario $Chal_{14}T_7$ and $Chal_{14}T_{14}$ and with regard to the antibody response (Figure 5.6d) between scenario $Chal_{35}T_7$ and $Chal_{35}T_{14}$. The potential influence of age at challenge on the evaluation of immunoresponsiveness is illustrated in the difference in the genotype ranking with regard to both the acute phase response (Figure 5.6a,b) and the antibody response (Figure 5.6c,d) between scenario $Chal_{14}T_7$ and $Chal_{35}T_7$ and between scenario $Chal_{14}T_{14}$ and $Chal_{35}T_{14}$.

5.4 Discussion

Based on current available knowledge on the immune system, we have developed a model that describes the development of immunocompetence and the kinetics of immunoresponsiveness in the face of an extra-cellular bacterial challenge in the young chick. In addition, we have demonstrated that the individual components of this model fit published experimental data well in most cases. A lack of fit of the equation for, for example, acute phase response, for some immunological variables was moreover to be expected. The acute phase response is an immune system component which covers a wide range of variables and it is, therefore, not realistic to expect that one very simple equation could adequately describe all scenarios. Simplicity as well as transparency must, however, have a trade-off with the accuracy, and for the ultimate goal of this model, small deviations from the modeled pattern are not expected to have a large impact on the global results and the inferences drawn from the model. The fact that the acute phase- and antibody response components fitted published experimental data well even when parameter input values were based on other published studies is promising for the validity of the model. Although the model did not fit published experimental data equally well in all cases, the choice of equations, therefore, seems appropriate. Unfortunately, it was not possible to evaluate the interrelations between the individual model components because of the sparsity of studies with sufficient experimental data. For the same reason, the predictive ability of the model cannot, as yet, be fully validated. Fitting of the modeled acute phase- and antibody response to experimental data were in

most cases based on parameter estimates obtained from separate studies, and these generally did indicate a good predictive ability of the model.

In some cases, deviations between model predictions and experimental data were observed. The standard errors on the experimental data, which in some cases are considerable (e.g. Burton and Harrison, 1969; Leitner et al., 1992; Jeurissen et al., 2000), were not taken into account, and this may explain the observed deviations. Moreover, some data were based on experiments with other pathogens than extra-cellular bacterial, because sufficient data with extra-cellular bacterial pathogens were not available. An effect of such pathogens interacting differently with the immune system cannot be excluded as a possible explanation for deviations in these data. Globally, however, effects of type of pathogen are mainly expected to be observed in the type of variables activated, e.g. in the baseline immunity and acute phase response, as well as in scaling, e.g. in the antibody response. Hence, such data are still considered informative for the global patterns of development of immunocompetence and the immunoresponsiveness kinetics. Further, moderate changes in immunological variables may not have an effect of actual biological significance on immunocompetence or immunoresponsiveness (Keil et al., 2001). Therefore, given the currently available experimental data, it is concluded that the predictive abilities of the individual model components are adequate. Potentially, the model may also be used as a generator of hypotheses on global immunological relationships to be tested experimentally.

To fully validate the model, a comprehensive study with experimental data is needed. Such data should include measurements of maternal antibodies and baseline immunity, as well as of acute phase- and antibody response. Model parameterization should then ideally be done with independent data, e.g. two halves of the dataset arising from the study. Experimental data from different studies, in which different populations of chickens have been used, is less suitable for validation, because the model parameters are unlikely to be universal, given the variation observed in immunological variables among different chicken genotypes and flocks (Kaleta and Siegmann, 1978; Jarosinski et al., 2002; Kaiser et al., 2003). For the ultimate goal of this model, i.e. to evaluate challenge and measurement strategies for selection purposes, universal parameters are not an absolute requirement, because selection is done within genotypes. The evaluation of challenge and measurement strategies should, therefore, also be done within genotypes. To allow for such an evaluation, a preliminary study could be done in which maternal antibodies and baseline immunity are measured in healthy chicks from a given genotype, to parameterize the model, and subsequently different challenge and measurement strategies could be investigated and evaluated.

In practice, utilization of the model not only requires parameterization, but also requires decisions on which immunoresponsiveness variable(s) it is desired to measure. Whereas measuring the maternal immunity and the antibody response is straightforward, defining which components of baseline immunity and the acute phase response to measure is not. With the model described in this study, suggestions are not made on which variables to measure. Amongst others, this will depend on the indicative value of such variables for health (the ultimate goal of selection for improved immunocompetence and/or immunoresponsiveness). The expression of the components in index values does, however, ensure that the model can be used regardless of which variable(s) are eventually measured. The baseline immunity may be parameterized based on a single variable, as in this study, or it may be parameterized based on a combination of variables of importance. The same is also true for the acute phase response.

Few existing mathematical models deal with the immune system in a broad scope (Wodarz and Nowak, 1999). Most have been designed to understand and explore specific molecular and cellular processes of the immune system development, or response or interactions between specific immune system components. For example, T-cell and macrophage interactions, T- and B-cell interactions, B-cell networks, and dynamics of primary and secondary antibody responses have been modeled. Others have been tailored to specific diseases, such as HIV or hepatitis virus (Kaufman et al., 1985; Chowdhury and Stauffer, 1990; Smirnova, 1991; Chowdhury, 1993; Morel, 1998; Wodarz and Nowak, 1999; Kleinstein and Seiden, 2000; Perelson, 2002).

The model in this study is therefore unique in its scope, covering non-specific immunity along with maternal immunity and humoral immunity. In the evaluation of immunocompetence or immunoresponsiveness all these parts of the immune system may be of importance, and the relatively broad scope is, therefore, an important part of the strength and usefulness of this model. Additionally, to our knowledge, the model in this study is the first to describe the development of immunocompetence and kinetics of immunoresponsiveness in young chicks; emphasizing the potential importance of the model. Moreover, the model in this study is relatively simple both in terms of the description of biological processes and the number of parameters that require estimation. This simplicity makes it easier to parameterise and easier to for use for the purpose of the evaluation of challenge and measurement strategies compared to existing models, which require a relatively large number of parameters (Faro et al., 1997).

To evaluate the immunoresponsiveness of three simulated broiler genotypes, four scenarios were compared by varying the experimental design with regard to challenge age and measurement timing. The comparison illustrated that the ranking of broiler genotypes with different immunocompetence development is expected to be different

depending on which type of immunoresponsiveness is measured, measurement timing, and age at challenge. The re-ranking of the broiler genotypes between the two challenge ages for the acute phase- and antibody response is caused by different rates of development of the baseline immunity between genotypes. Different development patterns of the maternal immunity also result in re-ranking of the antibody response, but less so the acute phase response. For the evaluation of challenge and measurement strategies for selection purposes, the risk of re-ranking is of major importance, and the development of both maternal- and baseline immunity must therefore be taken into account when evaluating these strategies. In practice, the challenge and measurement strategy where the largest differences between genotypes, groups, or individuals are expected to be observed, and where the immunoresponsiveness is expected to reflect the breeding goal as defined, e.g. peak, average, or fastest immune response, should be chosen. Hence, the risk of re-ranking is of importance whether it is desired to evaluate the actual ranking of genotypes or simply to illustrate the presence of differences between genotypes. With the risk of re-ranking because of the development of maternal- and baseline immunity, choosing the best challenge and measurement strategy becomes difficult. The model presented in this study provides a tool to predict immunoresponsiveness, taking this development into account and thereby aid in choosing the best challenge and measurement strategy.

An influence of the type of immunoresponsiveness measured on genotype, group, or individual ranking has also been found in many published studies on experimental data (Bacon et al., 1972; Lillehoj and Li, 2004; Withanage et al., 2005), but its importance is rarely fully recognized. For example, it has been found that there is genetic independence between selection for antibody response, cell-mediated immune response, and phagocytic activity (Pinard-van der Laan and Monvoisin, 2000). Thus, the ranking conclusions reached may depend on the trait measured.

An effect of measurement timing on genotype-, group-, or individual ranking is also observed in several published studies on experimental data, although the importance of this effect is rarely recognized. For example, Boa-Amponsem et al. (1999) compared two White Leghorn chicken lines that had been selected for high (HA) or low antibody response (LA) 5 days after a challenge with sheep red blood cells between 41 and 51 days of age. The lines were compared based on antibody response to a primary challenge with sheep red blood cells at 14 or 28 days of age. It was found that even though the HA line showed a greater antibody response 3 and 6 days after a primary challenge, the LA line showed an equally high antibody response after both a secondary and tertiary challenge. It was proposed that this was due to different genetic control of primary immune responses and immunological memory. However, re-ranking in the

development of baseline immunity or differences in the decline of maternal immunity may also provide an explanation, because selection was based on a measurement at a single time point. When the objective of the studies is not directly related to the kinetics of the immunocompetence or immunoresponsiveness, immunological variables are often measured at only one or perhaps two points in time. Ranking has been observed to change depending on measurement timing for maternal antibodies (Lemaire et al., 2000), lymphocyte development (Bar-Shira et al., 2003), the acute phase protein α1-acid glycoprotein (Takahashi et al., 1998), several cytokine responses (Kaiser et al., 2003), and antibody responses (Kreukniet and van der Zijpp, 1990; Koenen et al., 2002; Cheema et al., 2003). Each measurement is only a snapshot of reality, and as was illustrated in this study, conclusions on ranking based on only a single measurement cannot necessarily be reliably extrapolated if there is re-ranking of, or even differences in, immunocompetence development. The model in this study can take into account differences in immunocompetence development, and therefore it can assist in choosing the best timing of measurement(s) in the challenge and measurement strategy.

An influence of challenge age on genotype-, group-, or individual ranking is also observed in several published studies on experimental data. In the study of Boa-Amponsem et al. (1999), the differences in the primary antibody response between the HA and LA line were larger when the age of challenge was 28 days of age than when it was 14 days of age. This may be explained by differences in the development of maternal- or baseline immunity, and therefore illustrates the importance of challenge age for selection purposes. For example, there is re-ranking in macrophage function and antibody response (Koenen et al., 2002; Cheema et al., 2003), presumably due to development of the immune system. Whether the importance of this influence is recognized is unclear, because the challenge age is often not explicit or justified in publications. Sometimes the challenge age is based on the ages at which pathogen prevalence is assumed to be greatest in field data. At other times, the challenge age is chosen to avoid major effects of maternal immunity or to coincide with the so-called "window of susceptibility", where maternal immunity is fading and the chicks own immune system is still relatively undeveloped. However, this still defines a wide time period, e.g. from 2 weeks of age up to even 6 weeks of age. The model in this study can take into account differences in immunocompetence development, and predict the expected age at which maternal immunity has faded, and therefore, it can assist in choosing the best challenge age in the challenge and measurement strategy.

5.4.1 Conclusion

This model describes the development of immunocompetence in young chicks along with the kinetics of their immunoresponsiveness, and individual equations fit published data adequately. The model illustrated that experimental design (type of immunoresponsiveness measured, measurement timing, and challenge age) can have an important effect on the ranking of genotypes, groups, or individuals, and on the reliability of extrapolations based on this ranking. It is concluded that this model is a potential useful tool in the evaluation of challenge and measurement strategies for selection purposes, such as enhanced immunocompetence or immunoresponsiveness. Moreover, it may be used as a generator of hypotheses on global immunological relationships to be tested experimentally.

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Chapter 6: Modeling Variability in Immunocompetence and Immunoresponsiveness

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Abstract: The aims of this paper were two-fold: 1) to develop a stochastic model that reflects observed variation between animals and across age in immunocompetence and responsiveness; and 2) to illustrate consequences of this variability for statistical power of genotype comparisons and selection. The considerable amount of variability in immunological variables may cause non-normalities and heteroscedasticity in variance across age. A stochastic model of immunocompetence development and responsiveness kinetics was developed, to enable taking this variability into account in evaluation of challenge and measurement strategies for selection. The characteristics of the variation in model output reflect those observed in literature, to the extent that the variation in literature shows a consistent pattern. Actual knowledge of the true variation and patterns of variation in immunological variables is rather limited though. Due to causal relationships among the immunocompetence and responsiveness components, and due to the stochastic variation across time being correlated with an assumed lower limit of the repeatability, the model also creates correlations among these and within these across time. Correlations among components, as well as within components across time, were generally of similar size to estimates reported in literature. The model predictions on correlations among immune system components indicate that selection for improved health through improved immunocompetence or responsiveness may be difficult due to the sometimes low correlations among components as well as within components across time. The model also provides insight into experimental design. In practice, the statistical power of an experiment with a given challenge and measurement strategy to test a given hypothesis is often too low. The model predicts that problems with low power can be reduced by increasing challenge age in the experimental design, and that especially it should be confirmed or ensured that maternal immunity is absent when challenge is done. Repeatabilities of immunocompetence and responsiveness across time are also relatively low sometimes in practice. Consequently, the age at selection as well as the age at which information is gathered for selection must both be considered

carefully regarding the age at which the immunocompetence or responsiveness is desired to be improved, because it suggests that differences between animals change depending on measurement timing.

Keywords: *Immunocompetence, Immunoresponsiveness, Variability, Simulation, Experimental Design, Chicken.*

to be Submitted

6.1 Introduction

Variability in type, level, and kinetics of immunocompetence and responsiveness between individual animals is considerable (e.g. Jacobsen et al., 2004; Leitner et al., 1992). Immunocompetence may be defined as the immunological ability to resist and recover from infection (Owens and Wilson, 1999). Immunoresponsiveness is the ability to mount an immune response. For example, Leitner et al. (1992) observed within-line coefficients of variation (*CV*) from 2 to 230% for antibody response kinetics, and Kaiser et al. (2003) observed *CV* up to ~25% in within-line cytokine mRNA responses. The variability is caused by intrinsic factors, e.g. maturity, stress, circadian rhythms, nutritional status, and genetic variation (e.g. Coe and Lubach, 2005; Dayan, 1996; Petrovsky, 2001), as well as environmental factors, e.g. infection age, type and pathogenicity and time after infection (e.g. Beal et al., 2004; Xie et al., 2002a; Yonash et al., 1996). The variability may cause non-normalities in immunological variables and heteroscedasticity in variance across time. As a consequence, the expected statistical power of an experiment with a given challenge and measurement strategy or expected differences between phenotypically selected animals may not be achieved in practice. This may be an additional explanation of why expected improvements in health have not been achieved in selection experiments based on selection on immunological variables (Yunis et al., 2002).

Ask et al. (2007) developed a deterministic, dynamic model that describes immunocompetence development and immunoresponsiveness kinetics in chicks depending on extra-cellular bacterial challenge. This model was potentially useful for evaluation of challenge and measurement strategies for selection purposes, i.e. to improve the chance of successful selection for health through selection on immunological variables. The utility of the model will be improved, however, if it can also account for variability, typically seen in immune responses, because stochastic variation can have a substantial effect on the genetic evaluation of animals as well as the evaluation of challenge and measurement strategies.

The aims of this paper are therefore: 1) to expand the model developed by Ask et al. (2007) into a stochastic model that reflects observed variation between animals and across time; and 2) to illustrate the consequences of this variability on the statistical power of genotype comparisons as well as for selection.

6.2 Materials and Methods

6.2.1 Model Description

Details about the model that was used as the basis for this study are given in Ask et al. (2007), but the main principles will be summarized in the following. The model is deterministic and dynamic, describing the immunocompetence development and immunoresponsiveness kinetics of an individual chick from 0 to 56 d of age, depending on an extra-cellular, pathogenic bacterial challenge. Immunocompetence was reflected in the magnitude of the two immune system components, maternal immunity and baseline immunity. Maternal immunity consists of maternal antibodies and the baseline immunity of innate immunity, natural antibodies, and naïve lymphocytes. Immunoresponsiveness was reflected in the magnitude of the two immune system components, acute phase and antibody response. The acute phase response consists of, among others, acute phase proteins and fever, and the antibody response is a primary response mainly consisting of IgM. All the immune system components were expressed on a standardized scale from 0 to 1. The challenge and immune system components were described by equations that described their development or kinetics as a function of age as follows:

$$Chal(age) = chal_0 + a_{chal}.\exp[-\exp[-c.k_{chal}.(age - age_{chal})]],$$

where $Chal(age)$ is the challenge load experienced by the host at any given age; $chal_0$ is the initial challenge load; k_{chal} is the proliferation rate; a_{chal} is the proliferation limit; c is a scaling parameter; and age_{chal} is the age at challenge.

$$MI(age) = mi_0.\exp[-rmi(age).age],$$

where $MI(age)$ is the maternal immunity at any given age; mi_0 is the initial maternal immunity; and rmi is the degradation rate.

$$BI(age) = 1/[[1/[bi_0 - cbi(age)] - 1] + \exp[-kbi(age).age]] + cbi(age),$$

where $BI(age)$ is the baseline immunity at any given age; bi_0 is the initial baseline immunity; kbi is the development rate; and cbi is a constant, which in combination with bi_0 prescribes the asymptote, abi: $abi(age) = 1/[1/[bi_0 - cbi(age)] - 1] + cbi(age)$.

$$APR(age - age_{APR}) = s_1[-APR_a[s_2.(age - age_{APR})]^2 + APR_b[s_2.(age - age_{APR})]],$$

where $APR(age - age_{APR})$ is the acute phase response at any given age after its initiation; age_{APR} is the age at which the acute phase response is initiated and defined as:

$age_{APR} = age_{chal} + 10[1 - BI(age_{chal})^{0.5}]$; APR_a and APR_b prescribe the dependence of the magnitude of the acute phase response on challenge and baseline immunity, where $APR_a = chal_0.BI(age_{chal})$ and $APR_b = [1 + kchal]chal_0.BI(age_{chal})$; and s_1 and s_2 are scaling parameters.

$$ABR(age - age_{ABR}) = ABR_a.(age - age_{ABR})^{s_3} \exp[-s_4.(age - age_{ABR})],$$

where $ABR(age - age_{ABR})$ is the antibody response at any given age after its initiation; age_{ABR} is the age at which the antibody response is initiated and defined as $age_{ABR} = age_{APR} + 10\ln[s_2.(age_{APR} - age_{chal}) + 1]$; ABR_a describes the dependence of the antibody response on challenge, maternal immunity, and acute phase response, where $ABR_a = \exp[chal_0.achal].\exp[-MI(age_{ABR})^{0.5}].\ln[1 + APR_{max}]$; and s_3 and s_4 are scaling parameters.

In the face of a challenge, the degradation rate of the maternal immunity increases by a factor, F_{rmi}, which is defined as: $F_{rmi} = s_5.chal_0^{0.5}$, where s_5 is a scaling factor. The development rate and the expected mature level of the baseline immunity both increase by a factor, F_{BI}, which is defined as: $F_{BI} = 1 + APR_{max}^{0.5}$, where APR_{max} is the peak of the acute phase response: $APR_{max} = 0.25.[s_1.chal_0.BI(age_{chal}).[1 + kchal]^2]$.

Including Between-Animal and Stochastic Variation. From this previous described model, a stochastic model was developed so that it describes a population of chicks with variation between chicks and within chicks across age.

A population of a given size, N_P, is created by simulating individual chicks, *i*. The individual parameter values for the immunocompetence ($mi_{0.i}$, rmi_i, $bi_{0.i}$, kbi_i, abi_i, and cbi_i) and the individual age at which the acute phase response is initiated ($age_{APR.i}$) were drawn randomly and independently from normal distributions. The parameters were assumed to be uncorrelated. For example, $mi_{0.i} = \mu_{mi_0} + z_i[N(0, \sigma^2_{initial})]$, where μ_{mi_0} is the population mean; $\sigma^2_{initial}$ is the variance, and $z_i[N(0, \sigma^2_{initial})]$ are randomly and independently drawn values for each individual from a normal distribution with mean, 0, and variance, $\sigma^2_{initial}$. The cbi_i was calculated by solving the quadratic equation relating cbi_i to $bi_{0.i}$ and abi_i:

$cbi_i = 0.5\left(abi_i + bi_{0.i} - \sqrt{abi_i^2 + bi_{0.i}^2 - 2bi_{0.i}.abi_i + 4abi_i - 4bi_{0.i}}\right)$. The normal distribution was chosen, because of its ubiquitous role in biology given the Central Limit Theorem.

Environmental noise and within-animal variation across time is simulated by adding daily stochastic variation to the individual immune system components as follows:

$$C_i(age) = \overline{C}(age) + k.\left(C_i(age) - \overline{C}(age)\right) + z\left[N\left(0,(1-k^2).\sigma_C^2(age)\right)\right],$$

where $C_i(age)$ is the value of the individual immune system component (*MI*, *BI*, *APR*, or *ABR*) at any given age; $\overline{C}(age)$ is the mean of the immune system component at any given age; k is the correlation between the immune system component at two consecutive days; $\sigma_C^2(age)$ is the variance of the immune system component at a given age; and $z\left[N\left(0,(1-k^2).\sigma_C^2(age)\right)\right]$ is randomly and independently drawn value from a normal distribution with mean, 0, and variance, $(1-k^2).\sigma_C^2(age)$. Variation is added daily, i.e. each day from 0 to 56 days of age, hence a = 57 times, and to account for this in the correlation across time, the correlation, k, is replaced by $\sqrt[a]{r_C}$, where r_C is the correlation between component values at day 0 and day 56. Correlations across time are measures of the repeatability, i.e. the degree to which several measurements on one individual are similar. The heritability is the lower limit of the repeatability (Falconer and Mackay, 1996), and it is, therefore, considered a biologically sensible measure of the correlations across time. Hence: $r_C = h_C^2$, where h_C^2 is the heritability of the immunocompetence and immunoresponsiveness components.

6.2.2 Simulations

Observed Variation in Immunocompetence. The characteristics of the variation in the model will be illustrated by the means, *CV*, and skewness of the immunocompetence and immunoresponsiveness of a simulated population. The population was simulated with a size N_P = 50,000 individuals given a set of benchmark parameter values for immunocompetence parameters and challenge parameters (Table 6.1) as well as for the variation, assuming that the parameters were normal distributed (Table 6.2). The large population size was chosen to create stable outputs.

The sensitivity of the variability in the model was then explored, using the same population as a starting point, by changing the parameter values within ranges considered to be biologically sensible, given observed variation in published studies (Table 6.1 and 6.2).

The correlations (Pearson) of the immunocompetence and immunoresponsiveness components were also calculated across time and among the components at different ages and given varying challenge ages, given the benchmark values. Correlations were expected to appear because of the assumed correlations across time within components

when adding stochastic variation and because of the causal relationships between the components as described in the *Model Description*.

Table 6.1. Benchmark values and ranges of immunocompetence parameters[1], challenge age, age_{chal}[2], and challenge parameters[3].

		Mi_0	rmi	bi_0	kbi	abi	age_{chal}	$chal_0$	$kchal$	$achal$
Benchmark values		0.33	0.10	0.06	0.22	0.53	7	0.5	0.7	0.5
Range	Min	0.16	0.03	0.01	0.01	0.02	0	0.1	0.1	0.1
	Max	1.00	0.26	0.44	0.48	1.00	42	1.0	1.5	1.0

[1] The immunocompetence parameters include the maternal immunity parameters: mi_0 (the initial level at hatch) and *rmi* (the degradation rate) and the baseline immunity parameters: bi_0 (the initial level at hatch), *kbi* (the development rate), and *abi* (asymptote, prescribed by bi_0 and a constant, *cbi*).
The benchmark values of the immunocompetence parameters were estimated in Ask et al. (2007) and based on data from Islam et al. (2002). The estimation procedure was described in Ask et al. (2007). The range of the immunocompetence parameters are the minimum and maximum estimates from five longitudinal studies (Kaleta, 1972; Boa-Amponsem et al., 1997; Toro et al., 1997; and Sahin et al., 2001; Islam et al., 2002).
[2] The benchmark value of the age at challenge is set to 7 days of age, because this is a common choice for challenging young chicks (Ask et al., 2006). The challenge age is varied from 0 to 42 days of age.
[3] The challenge parameters are the initial challenge dose ($chal_0$), the proliferation rate (k_{chal}), and the proliferation limit (a_{chal}). The benchmark values and the range are chosen based on the median, minima, and maxima of the respective parameter value intervals (Ask et al., 2007).

Effect of Heteroscedasticity in Variance on Statistical Power. Statistical power is defined as the probability of rejecting a false null hypothesis. Using the immune system model, the effect of heteroscedasticity in variance across time, i.e. age as well as time after challenge, on the statistical power to detect a given effect size in

immunoresponsiveness between two genotypes was explored. The effect size is the smallest practically or biologically meaningful difference between two groups. As a reflection of two genotypes, two populations (*A* and *B*) with size N_P = 50,000 were simulated (Table 6.3). Simulation was done by applying benchmark values for the stochastic variance in immunocompetence parameters, the age at which the acute phase response is initiated as well as for challenge parameters (Table 6.1 and 6.2). From each genotype, a sample of size, $N = N_s$, was randomly drawn from a discrete uniform distribution $F \in [1{:}N_P]$ to test the null hypothesis, H_0: $d = \mu_{A.ABR} - \mu_{B.ABR} = 0$, where d is the effect size, and $\mu_{A.ABR}$ and $\mu_{B.ABR}$ are the expected means of the antibody response in genotype *A* and *B* respectively. The sample size, N_s, to detect a given effect size, d = 0.10 (medium effect size; Cohen, 1988), with a desired probability, $\alpha = 0.05$, that H_0 is rejected when it is correct, and a desired power, $1\text{-}\beta = 0.90$, was calculated based on the equation given by Fleiss (1986), which assumes normality and equal variances of samples. A variance, $\sigma^2 = 0.0025$, was assumed based on a *CV* of 10% and the median of the antibody response = 0.5, because a *CV* of 10% is a commonly observed *CV* in many quantitative traits. This resulted in a sample size, $N_s = 7$. The power to detect the effect size, d, was then empirically calculated from the *z*-value, which was obtained, using an approximation of the standard normal distribution by Abramowitz and Stegun (1968). The equation given by Fleiss (1986) was then applied to calculate the sample size that would have been needed to detect of the simulation output differences between genotypes *A* and *B* given the simulation output variances of genotypes *A* and *B* with a power of 0.90. Results were averaged over 2000 replicates, which produced stable results. The challenge age was varied from 0 to 42 days of age.

Table 6.2. Benchmark values and range of: the coefficient of variation, *CV*, based on which the initial variance of the distributions from which individual immunocompetence parameter values are drawn ($\sigma^2_{initial}$) is calculated[1], the *CV* of the age at which the acute phase response is initiated, age_{APR}[2], and the heritability of the immunocompetence and responsiveness[3]. A range of -50% to +100% for all benchmark values was considered biologically sensible.

		$CV(\sigma^2_{initial})$	$CV(\sigma^2_{age_{APR}})$	h^2_C
Benchmark values		10	18	0.25
Range	Min	5	9	0.125
	Max	20	36	0.50

[1] The benchmark value of the *CV* on which the initial variance of the distributions from which individual immunocompetence parameter values are drawn is based is 10%, because this is a commonly observed *CV* in many quantitative traits.

[2] The benchmark value of the *CV* of the age at which the acute phase response is initiated is based on experimental results from Glass et al. (2003).

[3] The heritabilities of immunological variables are mostly low to moderate (Cheng et al., 1991; Mallard et al., 1992; Yonash et al., 1996), and a benchmark value of 0.25 was therefore considered sensible.

Table 6.3. Values of the immunocompetence parameters[1] for the simulated genotypes (populations) *A* and *B*[2].

Genotype[1]	mi_0	*rmi*	bi_0	*kbi*	*abi*
A	0.33	0.10	0.06	0.22	0.53
B	0.41	0.10	0.08	0.22	0.66

[1] The immunocompetence parameters include the maternal immunity parameters: mi_0 (the initial level at hatch) and *rmi* (the degradation rate) and the baseline immunity parameters: bi_0 (the initial level at hatch), *kbi* (the development rate), and *abi* (asymptote, prescribed by bi_0 and a constant, *cbi*).
[2] The values of the immunocompetence parameters of genotype *A* are based on data from Islam et al. (2002). The estimation procedure was described in Ask et al. (2007). The values of mi_0, bi_0, and *abi* of genotype *B* differ with 25% from genotype *A*, whereas the values of *rmi* and *kbi* of genotype *B* do not differ.

Implications of Repeatability for Statistical Power. Using the immune system model, the effect of the repeatability across time, i.e. age as well as time after challenge, on the significance of differences between two samples of chicks, selected from a single population based on extreme phenotypes, is explored. From a simulated population of size, N_P = 200, with the same parameter values as population *A* (Table 6.3), the 10 chicks with the highest and lowest values of baseline immunity at 0 days of age were selected (i.e. equivalent to 5% each if the baseline immunity is normal distributed at this age). The probability to detect the differences between the two samples across age and time after challenge was then explored. The procedure was repeated 2000 times to obtain stable outputs of the probabilities across age. The age at selection was varied up to 56 days of age.

6.3 Results and Discussion

6.3.1 Observed Variation in Immunocompetence

In Figure 6.1, the characteristics of the variation in the model are illustrated by the means (Figure 6.1a), the log of the *CV* (Figure 6.1b), and skewness (Figure 6.1c) of the immunocompetence and responsiveness given benchmark values.

The pattern of simulated variability in maternal immunity across age reflected the pattern of observed variability in literature in some cases, where *CV* tends to first slowly and subsequently faster increase with age (Figure 6.2a; Kaleta, 1972, Toro et al., 1997). The pattern of simulated variability in baseline immunity across age reflected the pattern of observed variability in literature in some cases (Burton and Guion, 1968; Burton and Harrison, 1969), but in most cases there was no clear pattern of variability (Figure 6.2b; Dhabhar et al., 1995; Toro et al., 1997; Bar-Shira et al., 2003). The pattern of simulated variability in the acute phase and antibody response across time after challenge reflected the pattern of observed variability in literature in some cases. In both cases, *CV* tends to initially decrease with time after antigen exposure and subsequently (after the peak response) to increase again (Figure 6.2c, d; Trout et al., 1988; Kreukniet

and van der Zijpp, 1990; Leitner et al., 1992; Takahashi et al., 1998; Jarosinski et al., 2002; Xie et al., 2002b; Bar-Shira and Friedman, 2006).

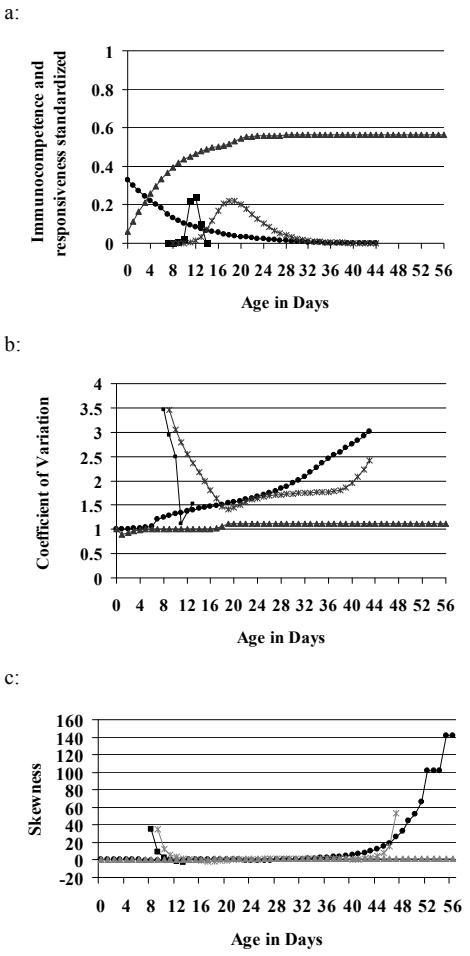

Figure 6.1. Means (a), the log of the *CV* (b), and skewness (c) of immunocompetence and immunoresponsiveness (—●—: maternal immunity; —▲—: baseline immunity; —■—; acute phase response; —✳—: antibody response).

The sensitivity to changes in the input parameter values, changes in the initial *CV*, challenge age and pathogenicity was investigated, and it was found that the patterns of *CV* and skewness across age were relatively insensitive, although there were scaling effects. Also in literature large ranges of *CV* were observed for maternal and baseline immunity as well as for acute phase and antibody response. The *CV* of maternal and

baseline immunity have been observed to range from 30 to 140% and from 8 to 40% respectively, and the *CV* for acute phase and antibody response have been observed to range from 29 to 61 and from 32 to 144% (e.g. Dhabhar et al., 1995; Yonash et al., 1996; Boa-Amponsem et al., 1997; Toro et al., 1997; Takahashi et al., 1998; Islam et al., 2002; Jarosinski et al., 2002; Xie et al., 2002b; and Bar-Shira and Friedman, 2006).

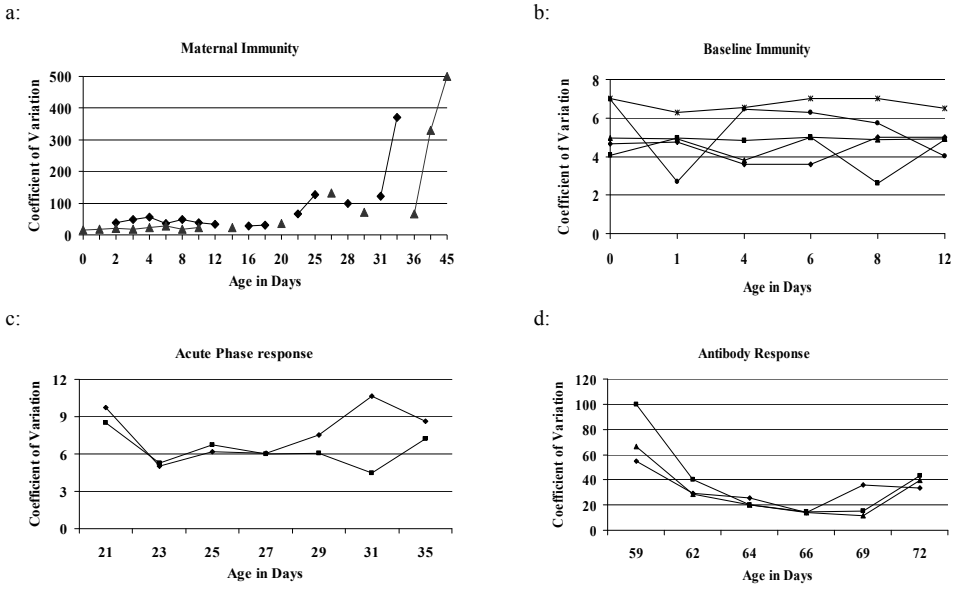

Figure 6.2. Examples of the pattern of *CV* across age of maternal (a; Kaleta, 1972) and baseline immunity (b; Bar-Shira et al., 2003) as well as of acute phase (c; Takahashi et al., 1998) and antibody response (d; Kreukniet and Van der Zijpp, 1990). The different lines are measured on different groups of chicks.

The pattern in simulated variability in baseline immunity, acute phase and antibody response did not always reflect the observed variability in literature, because in some studies, the observed variability varied randomly across age and time after antigen exposure (Cook et al., 1992; Parmentier et al., 1998; Whitanage et al., 2005). Such apparently unpredictable fluctuations may be caused by the relatively small sample sizes (e.g. n=3 to 14; Burton and Harrison, 1969; and n=3; Lillehoj and Li, 2004). The relatively small sample sizes in literature, in combination with these samples not always having been randomly drawn, brings up the important point that actual knowledge of the true variation and patterns of variation in immunological variables is rather limited. It was, therefore, also not possible to actually test the validity of the simulated variation patterns, but rather the simulated patterns of variation may be regarded as hypotheses for future investigations.

The sometimes apparently unpredictable fluctuations of variability across age and time after antigen exposure observed in literature may also be caused by non-normalities in the natural variation in immunological variables. Considerable skewness in immunological variables is the rule rather than the exception, and the skewness is not always removed by means of transformations (Bennet and Riley, 1992; McGuinness et al., 1997). For example, antibody levels (maternal as well as response to antigen exposure) as well as differentiated
white blood cell counts and acute phase protein levels are often considerably positively skewed (Odink et al., 1990; Islam et al., 2002; Moreno et al., 2003; Jacobsen et al., 2004; Joachim et al., 2004; Nauta, 2006; O'Neill et al., 2006). In our simulations, skewness generally agreed with this. Skewness was considerable for the maternal immunity, where the skewness increased gradually as the mean decreased. During the first week after challenge, the skewness became slightly negative (down to -0.17). Skewness for the baseline immunity remained positive and low, but it increased slightly following challenge. Skewness was also considerable for the acute phase response initially, but decreased as the response progressed, ranging from 35 to -2, and for the antibody response, the skewness was positive and high around initiation finalization of the response, but was otherwise low. In practice, skewness is particularly common when mean values are low, variances large, and values cannot be negative (Limpert et al., 2001), and these factors can also explain the positive skewness resulting from this model.

The stochastic simulation model is capable of creating a large amount of variation in immunocompetence and immunoresponsiveness, and given the causal relationships among the immunocompetence and immunoresponsiveness components, it has also created covariances among these, as reflected in the correlations (see Table 6.4).

Table 6.4. Correlations among immunocompetence and immunoresponsiveness components at selected time points[1].

	APR_{max}	ABR_{max}
MI(age=0)	-0.01	-0.30
MI(age=age_{chal})	-0.01	-0.58
BI(age=0)	0.02	0.03
BI(age=age_{chal})	0.72	0.37
BI(*age*=56)	0.23	0.12
APR_{max}	-	0.51

[1] *MI*(*age=0*) and *MI*(*age=age*$_{chal}$) are the maternal immunity *age* = 0 days of age and *age*$_{chal}$ (the age at challenge). *BI*(*age*=0), *BI*(*age=age*$_{chal}$), and *BI*(*age*=56) are the baseline immunity at *age* = 0 days of age,

age_{chal}, and 56 days of age. APR_{max} and ABR_{max} are the maximum acute phase and antibody response respectively.

Maternal immunity was uncorrelated to the acute phase response (peak), in accordance with their modeled independence (Ask et al., 2007). Maternal immunity was moderately, negatively correlated with the antibody response (peak) (-0.30 at hatch and -0.58 at the age of challenge), which has also been observed in literature (Moreno et al., 2003).

Baseline immunity at hatch was uncorrelated with the acute phase response as well as the antibody response, but the baseline immunity at the age at challenge was positively correlated with both the acute phase and the antibody response (0.72 and 0.37 respectively). In literature, only a few moderately positive (up to 0.32) correlations between baseline immunity and acute phase response have been reported, between white blood cell count or counts of various leucocyte sub-sets and the acute phase protein, α-acid glycoprotein (Clapperton et al., 2005). Other studies have, however, not been found, and other components of the baseline immunity might show different correlations. No literature on the quantitative relationship between baseline immunity and antibody response has been found. The model prediction of no correlations between the baseline immunity at hatch and the acute phase and antibody response in spite of the causal relationships prescribed by the model equations has important implications for selection. It has often been proposed to select for increased general immunocompetence to improve health (Mallard et al., 1998; Wilkie and Mallard, 1999; Pinard-van der Laan and Monvoisin, 2000). For this to be effective (result in genetic gain), immunocompetence must reflect immunoresponsiveness. The model predicts that effective selection for increased general immunocompetence to improve health may be difficult, because of the non-existent correlation between immunocompetence at one age and responsiveness at another. The acute phase and antibody response were moderately positively correlated (0.51 at peak), which is generally in accordance with the range observed in literature (generally low to moderate; Cheng et al., 1991; Kean et al., 1994).

The correlations among maternal immunity across age as well as antibody response across age (both pairwise between consecutive days) were moderately to highly positive (Figure 6.3). Moderately to highly positive correlations between measurements of antibody response at different ages has also been reported in literature (Yang et al., 1999). There is a small drop in the correlation in maternal immunity at the age of challenge (7 days of age), which is related to the effect of the challenge on the maternal immunity as reflected in the factor, F_{rmi}. There is a decrease in the correlations in antibody response related to an increasing average antibody response (the first drop in correlations illustrated in Figure 6.3) and related to the fading of the antibody response.

This is probably related to the non-linearity in the equation that prescribes the antibody response, which is larger when the antibody response is higher.

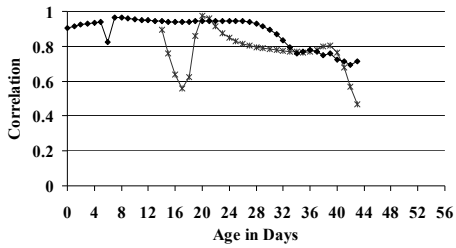

Figure 6.3. The correlations among maternal immunity ( ) and antibody response ( ) across age. The correlations are pairwise between consecutive days.

This has important implications for selection, because it stresses the need for more measurements across time to ensure a reliable measure of an individual's genetic value. The correlations among baseline immunity across age (pairwise between consecutive days) were high, and the correlations among acute phase response across age (also pairwise between consecutive days) were positive and low to high. Also in literature, generally moderate to high correlations (> 0.50) were reported for baseline immunity across age, e.g. for white blood cell count and counts of various leucocyte sub-sets measured at 1 week apart (Clapperton et al., 2005). Correlations among measurements of maternal immunity and acute phase response across age have not been found in literature.

6.3.2 Effect of Heteroscedasticity in Variance on Statistical Power

In practice, the expected statistical power of an experiment with a given challenge and measurement strategy to test a given hypothesis is often not achieved, because sample sizes are too small (e.g. Jennions and Møller, 2003; Maggard et al., 2003; Prentice, 2005). In spite of relatively large treatment effects, several published studies have not been able to show significance at all time points measured (Bacon et al., 1972; Cook et al., 1992; Jarosinski et al., 2002; Parmentier et al., 1998). Fluctuations, sometimes large, in variation across time may explain why the expected statistical power to test a given hypothesis is often not achieved and why sample sizes are too small. In Figure 6.4a, the power to detect the difference between two simulated genotypes, *A* and *B*, depending on challenge age and time after challenge is illustrated, and the sample size, which would have been necessary to detect the difference with a power of 0.90, is illustrated in Figure 6.4b. These results suggest that power first increases and thereafter decreases with time after challenge, the exact pattern depending on the challenge age. The necessary sample

size, like the power, changes over time after challenge and depends on the challenge age, but in all cases it rapidly increases to very high numbers. Contrary to expectance, however, the decrease in power and increase in sample size can largely be explained by the effect size and heteroscedasticity in variance appears to have little effect.

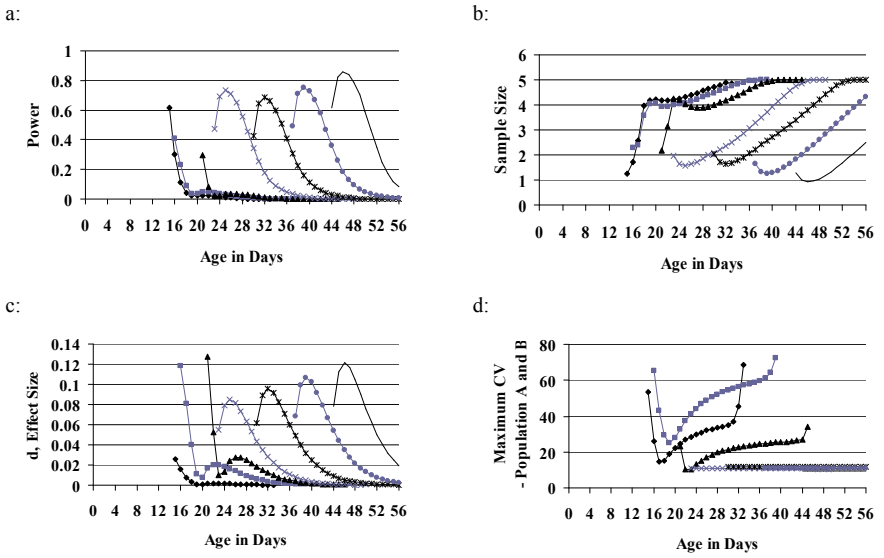

Figure 6.4. a: the power to detect the difference in antibody response between two genotypes *A* and *B* that differ by 25% in baseline immunity given a sample size of $N_s = 7$ and depending on challenge age as well as on age after challenge; **b:** the log of the sample size needed to detect the difference in antibody response between two genotypes *A* and *B* that differ by 25% in baseline immunity with a power of 0.90; **c:** the effect size, *d*, i.e. the difference in mean antibody response between population *A* and *B*, across age; and **d:** the maximum *CV* in antibody response of population *A* and *B* across age. The different curves reflect different challenge ages: : 0 days; : 7 days; : 14 days; : 21 days; : 28 days; : 35 days; : 42 days.

In Figure 6.4c, the effect size across time is illustrated, and the pattern is similar to that of the power across time. There was considerable heteroscedasticity in variance with a pattern across time different from that of the power and effect size (Figure 6.4d), yet it did not affect the power significantly. In the evaluation of challenge and measurement strategies for testing group differences, it is, therefore, important to account for heteroscedasticity in effect size, rather than variance, across time. The model predicts that the heteroscedasticity in effect size across time decreases when the challenge age increases and that problems with low power due to this heteroscedasticity will, therefore, be reduced with increasing challenge age. Especially, the model predicts that it is important to increase the challenge age to an age at which the maternal immunity is no longer of influence, because the maternal immunity causes relatively high fluctuations in

effect size across time. In practice, the choice of challenge age is sometimes based on avoiding major effects of maternal immunity. It is, however, rarely confirmed whether maternal antibodies have actually faded, and the actual age at which maternal antibodies have faded completely varies considerably (Smith et al., 1994; Jeurissen et al., 2000; Ahmed and Akhter, 2003), making it difficult to predict. The model predictions stress the importance of confirming or ensuring their absence before challenging is done.

6.3.3 Implications of Repeatability for Statistical Power

Phenotypic selection of animals at a given age, commonly used in immunological comparative studies, has not always lead to the expected phenotypic differences at later ages or at other times after challenge. In practice, repeatabilities of immunocompetence and immunoresponsiveness across time are in some cases relatively low, and this has important implications for selection strategies as well. In the simulation, the correlation across time of within animal variation was restricted to a minimum of 0.25. This was considered biologically reasonable, because a measure of the minimum repeatability of a given trait is the heritability of that trait (Falconer and Mackay, 1996), and many immunological variables have been observed to have moderate heritabilities (Cheng et al., 1991; Mallard et al., 1992; Yonash et al., 1996). For example, Siwek et al. (2006) reported heritabilities up to 0.42 for natural antibodies in chickens, and Henryon et al. (2006) estimated heritabilities for total and differential white blood cell count in pigs to between 0.22 and 0.30. Cheng et al. (1991) reported heritabilities up to 0.28 for phagocytosis and up to 0.53 for antibody response.

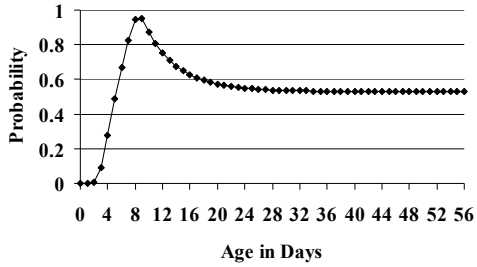

Figure 6.5. An example of the probability across age to detect a difference in baseline immunity at any given age between two groups of extreme individuals selected from the same population at 0 days of age.

In Figure 6.5, the probability across age to detect a difference between two groups of extreme individuals selected (phenotypically) from the same population at 0 days of age is illustrated. With increasing age at selection of the extreme groups of individuals, the average as well as minimum probability to detect a difference at another age is predicted to increase due to the maturation of the baseline immunity and, therefore, reduction in within-animal variation. This change in the probability to detect a difference at another

age than the selection age has important implications for future implementation of selection for improved immunocompetence or responsiveness, because it suggests that differences between animals change, depending on measurement timing. Therefore, selection for improved immunocompetence or responsiveness at any particular age may not result in the desired response at other ages. The age at selection as well as the age at which information is gathered for selection must, therefore, both be considered carefully regarding the age at which immunocompetence or responsiveness is desired to be improved.

6.3.4 Conclusions

In conclusion, a model has been developed that describes the development of immunocompetence and kinetics of immunoresponsiveness including between and within animal variation. This model reflects the characteristics of the considerable amount of variation observed in literature as far as this variation shows systematic patterns across time. Further, the model creates correlations among the immunocompetence and responsiveness components as well as within these across time. These correlations were generally of similar size to estimates reported in literature, and they indicate that selection for improved health through improved immunocompetence or immunoresponsiveness may be difficult due to the sometimes low correlations among components as well as within components across time. The model also provides insight into experimental design. In practice, the statistical power of an experiment with a given challenge and measurement strategy to test a given hypothesis is often too low. The model predicts that problems with low power can be reduced by increasing challenge age, and that it should be confirmed or ensured that maternal immunity is absent when challenge is done. Repeatabilities of immunocompetence and responsiveness across time are also sometimes relatively low in practice. Consequently, the age at selection as well as the age at which information is gathered for selection must both be considered carefully regarding the age at which immunocompetence or responsiveness is desired to be improved.

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Chapter 7: General Discussion

The main objectives of this dissertation were to evaluate broiler susceptibility to colibacillosis and the potential of genetic selection to reduce broiler susceptibility to colibacillosis. In this final chapter, the experimental design used in Chapters 2, 3, and 4, the main findings of this dissertation, and the most important hypotheses generated will be evaluated and discussed in relation to potential genetic selection to reduce susceptibility to colibacillosis in broilers.

7.1 Evaluation of Experimental Design – Recommendations for Genetic Evaluations

The experiment described in Chapters 2, 3, and 4 was designed with the purpose to identify the relative susceptibility to colibacillosis of different genotypes. A genotype refers to a population of chickens with the same genetic background, such as a pure-line or a crossbred. This design will be evaluated in the context of the results, newly acquired knowledge, and the issues of experimental design that were explored with the immune system simulation model described in Chapter 5, and recommendations for genetic evaluations will be given. Considerations of importance for the experimental design include decisions on the following: *number and type of genotypes*; *traits to measure and measurement timing*; *sample sizes*; *challenge age*; and *challenge procedure*.

7.1.1 Number and Type of Genotypes

In many studies in which genotypes are compared, just two or three genotypes are tested: one that is expected to be relatively non-susceptible, a second that is expected to be highly susceptible, and a third that is expected to be intermediately susceptible. In our case there was no prior knowledge on the susceptibility to colibacillosis of the available genotypes, and therefore a relatively high number of genotypes, namely eight, was tested. A choice of two or three of these genotypes could have been based on traditional expectations of the general susceptibility to disease. Traditional expectations are that sire lines are more susceptible to disease than dam lines, slow-growing lines are less susceptible to disease than both sire and dam lines, and crossbreds are less susceptible to disease than the pure-lines from which they descent. Based on these expectations, a logical choice of two or three genotypes would, therefore, have been a sire and a dam line, and either a crossbred or a slow-growing line. However, in our experiment the relative susceptibility of the tested genotypes did not comply to these traditional expectations (Chapter 3). This demonstrates that situations where prior knowledge is vague, testing a high number of genotypes is better than testing just two or three. Hence, more genotypes should always be tested, unless there is a specific reason for testing two

particular genotypes, such as the desire to evaluate the susceptibility of two genotypes divergently selected for high and low immune response. Our results also illustrate that extrapolations of results from one genotype to another is difficult.

7.1.2 Traits to Measure and Measurement Timing

Evaluation of susceptibility to disease can be based on clinical signs. The choice of which clinical traits to measure in our experiment was based on relative practical importance along with ease of measurement method. The timing of measurements in our experiment (Chapter 2) was limited by practical restrictions. Results from our experiment provided indications for which traits to measure and timing of measurement for future genetic evaluations. For example, feeding behaviour did not provide additional information on the susceptibility to colibacillosis (Chapter 2), and it should, therefore, be either omitted or measured more frequently in future genetic evaluations. Alternatively, individual feed intake should be included, because it is likely to provide additional information by virtue of it being an objective and quantitative trait. The indication that mortality could be ascribed to either of two causes, i.e. septicaemia or polyserositis (Chapter 2), suggests that daily registration of mortality is valuable information for susceptibility to colibacillosis. It was indicated that the severity of lesions was associated with the type of lesions (Chapter 2), and therefore, it is not necessary to score lesions in future genetic evaluations. Establishing whether there is airsacculitis or systemic lesions is sufficient. To ensure information on the growth of all challenged chicks, it is necessary to measure body weights daily after challenge. Body weights of chicks that died in our experiment showed that chicks either stopped growing or even lost weight during the last day before dying (Chapter 2). As a consequence, much information on the growth of chicks that die will be lost unless body weights are measured daily after challenge, because the body weight of dead chicks is mainly informative on the growth during the last day before dying and not the general growth following challenge.

7.1.3 Sample Sizes

In calculating the required sample size when designing the experiment, it was necessary to make assumptions on the mortality and the variation in measured traits. A mortality of 15 to 20% in the challenge group was assumed based on previous challenge experiments with commercial broilers of the same age and using the same challenge dose. However, the mortality in our experiment varied from 0 to 45.5% depending on genotype. At termination of the experiment, the sample size in the challenge group was therefore too small in four of the eight genotypes for at least some comparisons (mortality > 32%). For example in Chapter 4, differences of >20% were non-significant

between these genotypes. This demonstrates the importance of a prior estimate of mortality to a given challenge procedure within genotypes before beginning actual genetic evaluations. In general, the sample sizes were still considered sufficient though, because important significant differences between genotypes could still be detected (Chapter 3).

7.1.4 Challenge Age

Challenge was done in "young" chicks (1 week of age) with *E. coli* alone to provoke primary colibacillosis, in spite of the higher economic importance of secondary colibacillosis, which in practice occurs in "older" chicks (3 weeks of age or older) (Carlson and Whenham, 1968; Goren, 1981). The "young" challenge age was chosen because interpretation of susceptibility to secondary colibacillosis is complicated by primers. There are many possible primers, ranging from ammonia and dust to various viral infections. To fully evaluate the susceptibility to secondary colibacillosis, it would be necessary to challenge with a variety of primers, because the observed susceptibility may be a result of the primers or interactions between the primers and the *E. coli*. For this, a large number of groups, and thereby animals and isolators (due to the infectiousness of viral primers), would be required, and this would be difficult due to practical limitations (number of animals needed versus number of isolators available and the number of animals per isolator). Challenging at "young" age implies the assumption that susceptibility to colibacillosis at "young" age is positively correlated with susceptibility to colibacillosis "older" age. Although development of the immune system causes differences between susceptibility to colibacillosis in "young" and "older" chicks (Chapter 3), experimental results do suggest that primary colibacillosis *can* occur in "older" chicks: Matthijs et al. (2003) reported that up to 23% of chicks that were challenged with *E. coli* alone at 34 days of age had airsacculitis and up to 14% had systemic lesions. It is, therefore, possible that susceptibility to colibacillosis in "young" and "older" chicks is correlated. Yet, for future genetic evaluations, it is important to verify the ranking of genotype susceptibility at "older" age, and to estimate the genetic correlation between susceptibility at "young" and "older" age, because, in practice, colibacillosis causes the most losses at "older" ages. The ranking may change with age, for example due to the susceptibility to colibacillosis at "older" ages being associated with susceptibility to the primers as well. In literature, there is consensus that susceptibility to colibacillosis at "older" ages is associated with the primers as well as the *E. coli* (e.g. Bumstead et al., 1989; Gross, 1990; Nakamura et al., 1992; Peighambari et al., 2000). This indicates that susceptibility to colibacillosis is not the same trait at

different ages. This must be accounted for in a future breeding goal and traits to be recorded.

7.1.5 Challenge Procedure

The challenge procedure was chosen based on previous experiments in which it was shown to result in clinical signs similar to naturally occurring colibacillosis (Goren, 1978). It was a completely controlled challenge, as each chick was individually inoculated with the *E. coli*. A semi-natural challenge is preferable for selection purposes instead of a completely controlled challenge, because it will reflect the complexity of naturally occurring colibacillosis better. Semi-natural challenge testing is when the pathogen is introduced into the environment, for example by spraying, and infection is left to spread by itself. Semi-natural challenge testing is furthermore more informative for genetic evaluations than naturally occurring colibacillosis by virtue of a higher incidence of infection among measured chicks. In addition, compared to a completely controlled challenge, semi-natural challenge has the advantage of inoculation procedures no longer being an issue. There are also disadvantages of using a semi-natural challenge though. The spread of infection through the population is uncontrolled, and this complicates the optimal timing of measurements. Moreover, it is very difficult to standardize the challenge pressure, and hence to create an equal basis for the genetic evaluation of susceptibility to colibacillosis. However, if a challenge environment can be created in which the challenge pressure is permanently relatively high, all chicks should at least be exposed to challenge, thereby ensuring information on the susceptibility from all individuals. Some variation in the challenge pressure is not an issue for genetic evaluations as long as the number of replicates (between-line evaluation) or relatives (within-line evaluation) of selection candidates that are challenged is high enough. Overall, the advantages of semi-natural challenge outweigh the disadvantages, and for selection purposes (genetic evaluations), a semi-natural challenge with a relatively high challenge pressure should, therefore, be done.

The challenge in our experiment was done with a single pathogenic *E. coli* strain, but for selection purposes it is recommendable to challenge with a cocktail of pathogenic *E. coli*. Although a cocktail of pathogenic *E. coli* may exert a different infection pressure on broilers than a single pathogenic *E. coli*, a cocktail is expected to reflect variations in naturally occurring colibacillosis better than a single strain, because naturally occurring colibacillosis can be caused by a variety of *E. coli* strains.

7.2 Breeding Goal for Selection against Susceptibility to Colibacillosis

Desired genetic improvement in susceptibility to colibacillosis, i.e. the importance in the breeding goal, should be based on economic importance along with welfare concerns.

The economic importance and welfare concerns of susceptibility to colibacillosis is highest on commercial broiler level (see Figure 7.1 for the typical organization of broiler breeding). This is due to the major losses due to colibacillosis being a result of growth retardation and condemnations (Goren, 1991), the relatively low biosecurity level, and therefore, relatively high incidence of colibacillosis, as well as the absolute numbers of chicks being highest on this level. Therefore, the breeding for reduced susceptibility to colibacillosis should focus on reduction of the incidence of colibacillosis at commercial broiler level. If, as mentioned in Paragraph 7.1.4, susceptibility to colibacillosis is not the same trait at different ages, then focus should be on reduction of the incidence of colibacillosis at all ages.

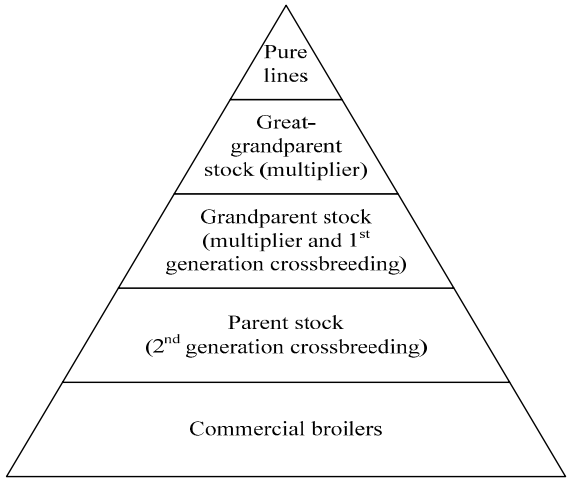

Figure 7.1. The typical organization of broiler breeding is a pyramid structure, where the pure-line broilers are at the top of the pyramid, and the commercial broilers are produced through a number of multiplier steps and crossbreeding steps. Typically there is therefore a time-lag of 4 generations between the genetic improvements achieved in the pure-lines and the expression of these improvements in the commercial broilers.

7.3 Traits to Measure for Selection against Susceptibility to Colibacillosis

Broiler susceptibility to colibacillosis was defined as a categorical trait with four exclusive categories based on pathological signs: 1) *chicks without lesions*; 2) *chicks with airsacculitis but no systemic lesions*; 3) *chicks with systemic lesions*; and 4) *chicks that die* (Chapter 2). This definition enables evaluation of broiler susceptibility to colibacillosis for future selection purposes, either as a trait on which selection can be performed or for evaluation of alternative traits to measure, because it was indicated that genetic variation is present in susceptibility to colibacillosis, and that selection is,

therefore, possible (Chapter 3). Potential traits to measure for selection against susceptibility to colibacillosis are indicator traits, i.e. traits that are genetically associated with the susceptibility to colibacillosis but can be measured in healthy chicks; clinical traits; or a combination of the two. In the following paragraphs (7.3.1 to 7.3.3) these alternatives will be explored further.

7.3.1 Traits measured on Healthy Chicks - Indicator Traits

Based on our experiment, it is possible to make suggestions about the biological background of the observed differences in susceptibility to colibacillosis. Knowledge on the biological background of the differences in susceptibility to colibacillosis is important, because it contributes to the identification of indicator traits to be recorded. Indicator traits may be used for selection against susceptibility to colibacillosis. The use of indicator traits circumvents the use of clinical traits, which has often been stated to be suboptimal (e.g. Uribe et al., 1995; Grandinson et al., 2002; Beaumont et al., 2003).

It was suggested that the biological background of the observed differences in susceptibility to colibacillosis were a result of differences in maternal antibodies or physical barriers in the respiratory tract (Chapter 2), e.g. cilia, mucus, and receptors. Examples of receptors are glycoprotein or glycolipid structures of the epithelial cells (Ramphal and Pyle, 1985). Many other factors may also play a role in susceptibility though. For example, natural antibodies; primary immune organs, e.g. spleen and bursa of fabricius; or supply organs, e.g. heart, liver, and lungs, may play a role in the susceptibility.

Additionally, other factors in innate immunity, such as cytokine response, macrophage activity, and acute phase protein response may play a role (e.g. Toth et al., 1988; Blackburn, 1994; Chamanza et al., 1999; Kim et al., 2001; Xie et al., 2002; Lillehoj and Li, 2004; Møller and Saino, 2004; Gruys et al., 2005; Whitanage et al., 2005). Although these components are dependent on antigen exposure, they can still be measured on healthy chicks and thereby abide by our definition of indicator traits.

The value of potential indicator traits should be based on a number of elements. Most importantly, potential indicator traits must have a high *predictive value*, i.e. *genetic correlation*, r_g, with the susceptibility to colibacillosis, as well as show *genetic variation* as reflected in a relatively high *heritability*, $h^2_{indicator}$. Based on these parameters it can be determined whether the correlated response in susceptibility resulting from selection on a indicator trait or a combination of indicator traits is higher than the direct response in susceptibility by selection on clinical traits (Falconer and Mackay, 1996). In the evaluation of potential indicator traits, it is further important to determine the dependency on *antigen exposure* (in vitro or vaccination) along with *antigen specificity*.

Costs of measurements are also of importance, but will not be considered in this discussion. Table 7.1 summarizes strong and weak points of a range of potential indicator traits. This will be discussed in the following sections.

Table 7.1. Strong and weak points[1] of potential indicator traits for susceptibility to colibacillosis for a number of elements of importance for indicator traits. Indicator traits are defined as traits that are genetically associated with the susceptibility to colibacillosis but can be measured in healthy chicks. Antigen exposure dependency does not imply infection as such, but the need for vaccination or in vitro testing. The scoring is based on published studies.

Potential Indicator Traits[2]		Predictive value / Genetic correlation	Genetic variation / Heritability	Antigen exposure and specificity
Maternal antibodies	Blood	÷	++	+++
	Respiratory	++	?	++
Natural antibodies	Blood	÷	+	+++
	Respiratory	++	?	++
Physical barriers	Ciliary activity	++	?	÷
	Mucus production	?	?	÷
	Receptors	++	+++	+++
Organ weights	Heart; liver; lungs	?	++	÷
	Spleen	?	+++	÷
	Bursa of fabricius	+	+++	÷
Cytokine responses	E.g. IL-x; INF-γ	?	+++	+
Macrophage activity	E.g. Nitric oxide production; phagocytic activity; carbon clearance	+	+	+
Acute phase proteins responses	E.g. OTF; SAA; α1-AGP	?	++	+

[1] The following signs are used to indicate strong and weak points of the potential indicator traits: ?, ÷, +, ++, +++. The '?' indicates that no published information has been found on the given element for the given indicator trait; the '÷' indicates affirmation for the given element and indicator trait, i.e. that it euthanization is not necessary; the '+', '++', and '+++' indicate increasing degrees of conformation for the given element and indicator trait, i.e. that euthanization is necessary.

[2] Maternal antibodies can be found in either the blood or in the respiratory tract in the intra-cellular fluid; Natural antibodies can be found in the blood and presumably also in the respiratory tract; Physical barriers are structures in the respiratory tract; IL-x (interleukins 1, 6, 12, etc.), and INF-γ (gamma interferon) are examples of cytokines; OTF (Ovotransferrin), SAA (serum amyloid A), and α1-AGP (α1-acid glycoprotein) are examples of acute phase proteins.

Predictive Value / Genetic Correlation. The role of maternal antibodies in infectious diseases has been shown in several published studies (Smith et al., 1994; Hassan and Curtiss, 1996; Ahmed and Akhter, 2003; Sahin et al., 2003). In our experiment, a role of maternal antibodies in the resistance to colibacillosis (Chapter 2) was not supported by the specific maternal antibody levels in the plasma (Chapter 4). Still, the role of maternal antibodies cannot be ruled out, because maternal antibodies in the respiratory tract

(measured in lavage fluid from respiratory system washings) rather than in the blood may play a role (Mondal and Naqi, 2001). On the one hand this makes sense, because maternal antibodies in the blood can only be of importance in the resistance of systemic infections, whereas maternal antibodies in the respiratory tract can be of importance for all respiratory infections. On the other hand, however, it is not known how maternal antibodies end up in the respiratory tract. Possibly, they are acquired through the blood in a concentration dependent manner in the same way as maternal antibodies in the blood and the egg yolk are related (Jeurissen et al., 2000). If this is the case, it seems unlikely that maternal antibodies in the respiratory tract would play a role in susceptibility to colibacillosis given that differences in maternal antibodies in the blood do not play a role.

Natural antibodies have previously been shown to play a role in susceptibility to both bacterial and viral infections, including respiratory infections (Ochsenbein et al., 1999; Ochsenbein and Zinkernagel, 2000; Baumgarth et al., 2005; Star et al., 2007). However, our study does not provide evidence for the role of natural antibodies in the susceptibility to colibacillosis. The measurements of antibodies in our study did not distinguish between natural and maternal antibodies, and we found no association between the amount of maternal antibodies in the blood and the susceptibility to colibacillosis. Still, the role of natural antibodies cannot be ruled out, because, like maternal antibodies, natural antibodies may also occur in the respiratory tract and thereby play a role in the susceptibility to colibacillosis. In contrast to maternal antibodies, natural antibodies may be independently produced in the respiratory tract, because they are produced by a subset of long-lived, self-replenishing B-cells (Baumgarth et al., 2005). Organized lymphoid structures called bronchus-associated lymphoid tissue exist in the avian respiratory tract, and these contain large numbers of antibody producing B-cells, which migrate there already during the first week after hatch. Further, antibody producing cells have been identified in interstitial lung tissue in chicks as young as 5 days of age (Reese et al., 2006). Therefore, it is possible that natural antibodies play a role in the defence against infection with *E. coli* in the respiratory tract.

In support of a role of physical barriers in protection against colibacillosis, the activity of cilia has been shown to prevent adherence of *E. coli* in the trachea of chicks (Ficken et al., 1987; Vidotto et al., 1990). Mucus in the respiratory tract functions as trapping mechanism of foreign material and as a transportation medium, and the functionality of ciliary activity depends on a sufficient mucus production (Richardson and Peatfield, 1980). Mucus production may therefore also play a role in protection against colibacillosis. In pigs, the absence of the K88 receptor in the small intestine (a virulence

factor of *E. coli*) provides close to 100% protection against intestinal colibacillosis (Rutter et al., 1975; Madec et al., 2000). Likewise, receptors in the respiratory tract in chicks may play a role in susceptibility to colibacillosis, because receptors are necessary for adherence of *E. coli*, and adherence of *E. coli* is a condition for colonization by *E. coli* (La Ragione et al., 2000).

There is only limited evidence of the role of other factors in the innate immune system in susceptibility to colibacillosis, e.g. cytokine levels, macrophage activity, and acute phase response, but as part of the immune response they do play a role in resistance to disease.

The bursa has been shown to play a role in susceptibility to colibacillosis by a comparison between bursectomized and non-bursectomized chicks (Sadler and Edgar, 1969), but the genetic correlation of organ weights with disease susceptibility has not been examined to our knowledge. Yet, organ weights are often used as indicators of general immunocompetence in, for example, toxicological research (Potter and Xu, 2001; Galloway and Handy, 2003; Fairbrother et al., 2004), implying a presumed role in disease susceptibility. For example, a heavy spleen has traditionally been associated with high immunocompetence, especially in chicken because of the absence of lymph nodes (John, 1994; Morand and Poulin, 2000; Møller and Erritzøe, 2000). Biologically, it would make sense that liver, heart and lung weights play a role in the susceptibility to colibacillosis, given that their weight is correlated with their respective functions. For example, gluconeogenesis in the liver ensures energy and nutrient availability for the growth and products of immune system cells (Beisel, 1975; Klasing and Johnstone, 1991). Likewise, a role of spleen and bursa weights in the susceptibility to colibacillosis makes sense. The spleen is responsible for lymphocyte production, maturation, and storage, antibody synthesis, and is also a site for phagocytosis of damaged cells and antigens (John, 1994; Smith and Hunt, 2004). The bursa functions as a site of lymphocyte maturation with a net flux of lymphocytes towards infected areas during infection (Challey, 1962; Nakamura et al., 1986; Glick, 1995). Therefore, given that organ function is associated with organ weight, all these organ weights are expected to have a predictive value for, i.e. genetic correlation with, the susceptibility to colibacillosis.

Summarizing, the maternal and natural antibodies *in the blood* can be eliminated as potential indicator traits, because of their (expected) low to non-existent predictive value for susceptibility to colibacillosis. A conclusion regarding the traits for which the predictive value is unknown cannot be drawn, but especially the traits that are measured in the respiratory tract (natural antibodies, ciliary activity, and receptors for *E. coli*) are expected to have relatively high predictive values.

Genetic Variation / Heritability. In most of the proposed indicator traits, the indication of presence of genetic variation have been found as reflected by line differences, and for several traits, heritabilities have been estimated, although not always in chickens. However, genetic variation in maternal and natural antibodies in the respiratory tract, cilial activity, and mucus production has to our knowledge not been studied. It is expected that maternal antibodies in the respiratory tract do show genetic variation though, given that maternal antibodies in the blood show genetic variation, and considering the expected association based on concentrations (Paragraph 7.3.1; *Predictive Value / Genetic Correlation*). Genetic line differences in chickens have been found for maternal antibodies in the blood, indicating a genetic component (Bumstead et al., 1993; Boa-Amponsem et al., 1997), and Grindstaff et al. (2003) suggested a moderate heritability of maternal antibodies in cows. Hence, a genetic component in maternal antibodies in the respiratory tract is also expected. Epithelial receptors for bacteria, such

Table 7.2. Heritability estimates of potential indicator traits published in literature.

Potential indicator trait	Heritability	Reference(s)
Natural antibodies in blood	0.11±0.07 and 0.42±0.14	Siwek et al., 2006
Cytokine response (IFN-γ)	0.72±0.22 and 0.85±0.11	Höhler et al., 2005
Macrophage activity (carbon clearance)	0.06 and 0.28	Cheng et al., 1991; Pinard-van der Laan and Monvoisin, 2000
Acute phase protein responses (C-reactive protein)	0.24±0.08 and 0.46±0.08	Best et al., 2004
Heart weight	0.19±0.06; 0.22±0.08; 0.23±0.06; 0.30±0.08; and 0.62	Toelle et al., 1991; De Greef et al., 2001; Rance et al., 2002
Liver weight	0.08±0.06; 0.17±0.06; 0.36; and 0.44	Toelle et al., 1991; De Greef et al., 2001; Rance et al., 2002
Lung weight (Total of both lungs)	0.23 and 0.34	De Greef et al., 2001
Bursa weight	0.78	Muir and Jaap, 1968
Spleen weight	0.51±0.09 and 0.54±0.09	Rance et al., 2002

as the K88-receptor in the pig intestine, have a genetic background (Edfors-Lilja, 1991), and likewise receptors in the respiratory tract have a genetic background. For the other potential indicator traits, heritabilities have been estimated in literature (Table 7.2), and they all show genetic variation in chicken, although heritability estimates for acute phase protein or cytokine responses in chicken have not been found.

Summarizing, all traits, except for those in the respiratory tract, i.e. maternal and natural antibodies, ciliary activity, and mucus production, showed genetic variation, as indicated by moderate to high heritabilities. Selection on these traits is, therefore,

possible. Genetic variation in the traits in the respiratory tract is to our knowledge unknown, but is certainly expected to be present.

Antigen Exposure and Specificity. The dependency of indicator traits on antigen exposure is important to consider, although it does not imply the need for infection or challenge testing, because this can be circumvented by in-vitro assays, for example, for measuring cytokine response, or by using vaccination instead of infection. However, either way it adds a complexity to the dependence on methodology, contingent in-vivo interactions are not accounted for, and vaccinations are undesirable, because even though they are not pathogenic they may still have an effect on the measurements of other traits of importance, e.g. growth. Antigen specificity is undesirable, because *E. coli* is a very complex pathogen with a large variety of virulence factors, or in other words antigens (e.g. Vidotto et al., 1997; Dho-Moulin and Fairbrother, 1999; Mellata et al., 2003). Moreover, antigen *non*-specificity has the advantage of potentially having a predictive value for completely other diseases that are of interest in broiler breeding programs. Cytokine and acute phase protein responses as well as macrophage activity are therefore more complicated as indicator traits than the rest of the proposed indicator traits.

In the future, complications due to dependency on antigen exposure may be partly circumvented through applying mathematical modeling to estimate breeding values for immunoresponsiveness. The model described in Chapters 5 and 6 provides a starting point for a framework to estimate the phenotypic immunoresponsiveness of individual selection candidates based on phenotypic immunocompetence measured on the selection candidates themselves, thereby circumventing antigen exposure. This model takes account of some interactions and non-linearities in the relationship between immunocompetence and immunoresponsiveness. Moreover, it can be used to model a range of different types of antigens and antigen doses, thereby accounting for antigen dependency to some extent. Although it poses a huge challenge, a more detailed expansion of the model may enable reliable estimation of individual breeding values for immunoresposiveness in the future.

Potential Indicator Traits. Taking into account all the considered elements, i.e. predictive value / genetic correlation, genetic variation / heritability, dependency on antigen exposure, and antigen specificity, a small number of potential indicator traits for susceptibility to colibacillosis stand out. Organ weights, especially bursa of fabricius, are promising indicator traits due to their moderate to high heritabilities, antigen independence and non-specificity. Further, the organ weights are expected to have a predictive value considering their functions. Ciliary activity is also a promising indicator

trait due to its potential predictive value, antigen independence and non-specificity. Furthermore, the level of natural antibodies in the respiratory tract is suggested to pose the most promising indicator trait. Natural antibodies in the respiratory tract potentially have a predictive value, are expected to show genetic variation, do not require antigen exposure, and have a relatively low antigen specificity.

7.3.2 Traits measured on Challenged Chicks - Clinical Traits

In Paragraph 7.3.1, the use of indicator traits for indirect selection against susceptibility to colibacillosis was suggested as advantageous relative to selection on clinical traits, because heritabilities of clinical traits are often low (Flock, 1993; Uribe et al., 1995; Janss and Bolder, 2000; Grandinson et al., 2002; Beaumont et al., 2003), and the necessity for challenge testing. However, heritabilities of clinical traits are not necessarily lower than those of indicator traits, and the necessity for challenge testing is not an argument against selection on clinical traits. Further, although the use of clinical traits for selection is basically a black-box approach, it has the advantage of reflecting susceptibility to colibacillosis directly according to the definition (Chapter 2).

The heritability of the quantitative clinical signs, such as growth retardation, is likely to be higher than in categorical clinical signs, such as mortality (Detilleux, 2001) and in that case, direct selection may, therefore, still result in higher genetic gain than indirect selection. Moreover, the genetic gain obtained from indirect selection is highly dependent on the genetic correlation between the indicator trait and the susceptibility to colibacillosis (Paragraph 7.3.1; Falconer and Mackay, 1996), which is not necessarily compensated by the higher level of heritability of the indicator trait.

Challenge testing is not necessarily needed for direct selection, because information may be obtained through naturally occurring colibacillosis. Naturally occurring colibacillosis in pedigreed chicks is, however, not likely to provide much information as pedigreed chicks are often kept under highly hygienic circumstances. Alternatively, relatives can be kept under commercial hygiene standards with a higher general infection pressure. However, some form of challenge testing is probably needed to obtain a sufficient amount of information for accurate genetic evaluations. Moreover, indirect selection requires (re-)estimation of genetic correlations between indicator traits and susceptibility to colibacillosis at regular intervals (once every 5 years), and to estimate these correlations challenge testing is necessary.

For selection on clinical traits, trait definitions, as well as recording methods, must be considered. In Chapter 2, a distinct pattern of mortality in response to an *E. coli* challenge was observed. This pattern has also previously been described (Ardrey et al., 1968; Matthijs et al., 2003), and it was suggested that it reflects differences in

susceptibility to colibacillosis. This should be utilized for selection through survival analysis, which is a method that takes the temporal nature of measurements (mortality) into account (Kleinbaum and Klein, 2005). Survival is an easily measured trait, but variation in mortality/survival will be relatively limited unless the challenge is relatively severe.

The subjective and categorical nature of lesions along with the association between lesions and disease development make the information that lesions provide relatively unreliable, inaccurate, and sensitive to measurement timing. Selection response based on selection on a combination of survival and lesions is, however, likely to be higher compared to selection on survival alone, simply because it provides additional information on differences in susceptibility to colibacillosis.

Alternatively, organ weights in response to challenge would pose more reliable, accurate, and less measurement timing sensitive, traits for selection given their objective and quantitative nature.

Organ weights (heart, liver, lungs, spleen, and bursa) were measured in our experiment. The organs were weighted in all control chicks as well as in the chicks in the challenge group that survived until the end of the experiment (at 14 or 15 days of age). In Table 7.3, the least-square means of the organ weights *±SE* in g are shown in the control and challenge group as well as in the challenge group depending on susceptibility to colibacillosis.

Challenge had a significant effect on the weights of the heart, liver, spleen, and bursa, but not on the lungs. Likewise, a significant effect of the susceptibility to colibacillosis was found on all the organ weights except for the weight of the lungs. This suggests that responses in heart, liver, spleen, and bursa weights are associated with response to challenge, and that organ weights indeed reflect susceptibility. Heart and liver weights were significantly higher in the *chicks with systemic lesions* (pericarditis and perihepatitis) than in all other chicks in both the challenge and control groups. The *chicks with systemic lesions* often had lesion scores of 3 (Chapter 2), which means that organs are covered with fibrinous exudate, and probably this is a major cause of the increased heart and liver weights in these chicks. This suggests that the increased heart and liver weights are products of disease rather than causal of differences in susceptibility to colibacillosis. The presence and amount of fibrinous exudate on the heart, liver, and air sacs is the basis of the evaluation of lesion scores (Chapter 2). Considering that fibrinous exudate must have an influence on the organ weights, then pericarditis and perihepatitis should also be reflected in the heart and liver weights. Likewise, airsacculitis may be reflected by air sac weights in response to challenge. The

procedure of removing the air sacs is very troublesome though. In conclusion, organ weights should replace lesions in a future selection strategy.

Table 7.3. Least-square means of organ weights (heart, liver, lungs, spleen, and bursa) *±SE* in g in the control and challenge group are shown along with the least-square means of the organ weights *±SE* in g in the challenge group depending on susceptibility to colibacillosis. An analysis of variance was used to analyse the data, adjusting for the fixed effects trial, treatment, sex, genotype, and age at measurement (14 or 15 days). Body weight at 14 or 15 days of age was included in the model as a covariate. Different letters within columns are indicative of significant ($P \leq 0.01$) differences.

	Heart	Liver	Lungs	Spleen	Bursa
Control	2.48*±0.03*a	13.02*±0.11*a	2.16*±0.02*a	0.31*±0.01*a	0.61*±0.01*a
Challenge, total	2.57*±0.02*b	13.90*±0.12*b	2.15*±0.02*a	0.40*±0.01*b	0.53*±0.01*b
Susceptibility to colibacillosis:					
No lesions	2.40*±0.03*a	12.96*±0.13*a	2.17*±0.02*a	0.35*±0.01*b	0.56*±0.01*b
Airsacculitis only	2.41*±0.07*a	13.27*±0.31*a	2.15*±0.06*a	0.40*±0.02*b	0.55*±0.03*ab
Systemic lesions	3.12*±0.05*c	16.84*±0.23*c	2.08*±0.04*a	0.53*±0.02*c	0.43*±0.02*c

Growth retardation potentially carries a higher amount of, and more reliable information on, the susceptibility to colibacillosis than survival or organ weights under challenge condidtions, simply because it can be measured objectively and on almost all chicks. Moreover, growth retardation is simple to measure, because it can simply be selected against by selecting for increased body weight or growth under challenge conditions. For these reasons, it is also likely that growth retardation has a higher heritability than survival. There are, however, indications that selection against growth retardation alone may result in *increased* susceptibility to colibacillosis, because growth retardation may be necessary to resist and recover from disease. This is because growth retardation is a product of the immune response through a combination of changes in amino acid, carbohydrate and lipid metabolism, increased basal metabolic rate, fever, and feeding inhibition/anorexia (e.g. Beisel, 1975; Klasing and Johnstone, 1991; Sonti et al., 1996). In our study, an incomplete association between clinical colibacillosis and growth retardation (*chicks with airsacculitis but no systemic lesions* did not show growth retardation; Chapter 2) indicated that growth retardation is *not* necessary to resist and recover from disease. Therefore, the use of variation in growth retardation for selection against susceptibility is recommended. In fact, a relatively high genetic gain in susceptibility to colibacillosis may be obtained from selection purely against growth retardation.

7.3.3 Combining Forces

There are no indications of mutual exclusiveness of the potential indicator traits proposed in Paragraph 7.3.1 and the clinical traits (Paragraph 7.3.2) with regard to explaining variation in susceptibility to colibacillosis. The broad range of virulence factors of *E. coli* (e.g. Vidotto et al., 1997; Dho-Moulin and Fairbrother, 1999; Mellata et al., 2003) implies a complex background of the susceptibility to colibacillosis. As each of the virulence factors of *E. coli* requires differential host defense mechanisms (Mallard et al., 1998), it makes sense that all the potential indicator traits and clinical traits simultaneously play a role in explaining the susceptibility to colibacillosis. Hence, a combination of traits is likely to explain variation in susceptibility to colibacillosis to a higher extent than single indicator traits or clinical traits. With respect to animal welfare, combined indirect and direct selection is also considered optimal relative to using information from either of them alone, because in either case challenge testing is necessary, and combining the two, therefore, allows for maximization of the use of information. Therefore, it is suggested that selection against susceptibility to colibacillosis should be done by combining the forces of indicator traits and clinical traits, for example, in an index.

Indeed, previous studies of the predictive ability for health of indices, including variables such as acute phase responses, spleen weight, phagocytosis, and macrophage nitric oxide production, have identified indices explaining up to 60% of the variation in resistance (Toussaint et al., 1995; Keil et al., 2001). Possibly, even higher amounts of variation could have been explained in this experiment if measurements had been taken at more than one time point. Information from different time points may in total provide more information on variation in resistance, considering that measurements at different time points may be evaluated differently (Chapter 5). It should be noted though, that these previous studies were based on phenotypic rather than genetic studies, and for selection, it is the genetic correlation between such an index and the susceptibility to disease which matters.

An additional advantage of an index is that it can take unfavorable associations among traits into account and thereby maximize desired response while at the same time minimizing undesirable effects (Stear et al., 2001). This is, for example, an advantage if growth retardation is included in the selection strategy, because growth retardation is not simply a product of disease but also a product of the immune response (Paragraph 7.3.2). By using an index that includes both growth retardation and traits that reflect immunoresponsiveness, contingent negatively correlated responses in immunoresponsiveness to selection against growth retardation can be avoided.

In conclusion, selection on clinical traits is recommended for selection against susceptibility to colibacillosis until the usefulness of the potential indicator traits (Paragraph 7.3.1) has been verified, after which a combination of indirect and direct selection is recommended. Relatives of selection candidates need to be subjected to this semi-natural challenge.

7.4 Information Sources and Organization of Data Collection

Potential information sources of indicator traits are: pure-line selection candidates; pure-line and crossbred sibs; and pure-line and crossbred progeny of selection candidates at various levels of the organization of a typical broiler-breeding program (Figure 7.2). Remembering that the breeding goal is to reduce the incidence of colibacillosis at commercial broiler level, the highest genetic gain is likely to be achieved based on traits recorded under commercial conditions. The fact that commercial broilers are crossbreds is also important for the potential genetic gain though. The reason for this is that when the breeding goal is the crossbred performance (in this case susceptibility to colibacillosis) then the genetic gain is higher based on crossbred information even when the genetic correlation for a given trait in the breeding goal between crossbreds and pure-lines is high, because crossbred information is basically added sib information

(Bijma and Van Arendonk, 1998). Crossbred information additionally allows for taking into account non-additive genetic effects, such as dominance and epistasis, which cannot

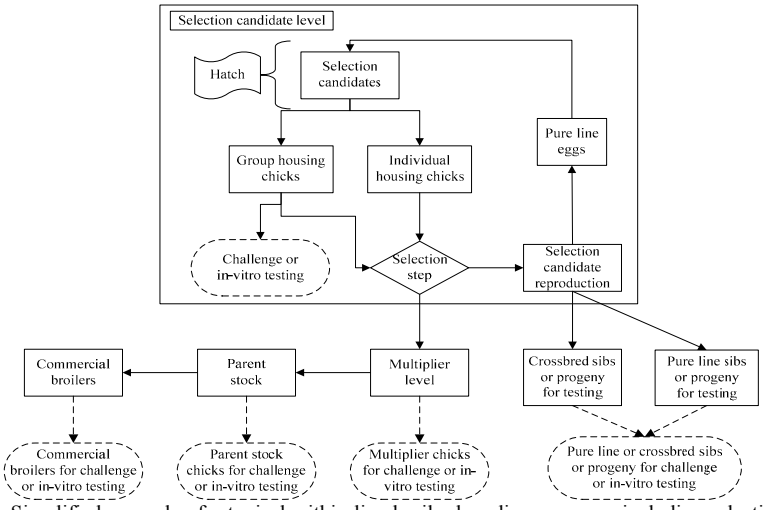

Figure 7.2. Simplified example of a typical within-line broiler breeding program, including selection steps at pure-line broiler level and various information sources. The places at which euthanization for in-vitro tests or challenge tests may be done are indicated by stippled ellipses.

easily be done based on pure-line information, because predictions cannot easily be made (Falconer and Mackay, 1996). Heterosis, which could be a result of such dominance or epistatic effects (Falconer and Mackay, 1996), is generally expected for fitness traits (Deng and Fu, 1998), such as susceptibility to colibacillosis. The importance of heterosis is reflected in the genetic correlation between the pure-lines and the crossbred, with a lower correlation indicating a higher importance of heterosis. Even for fitness traits, heterosis is not always positive (Wei, 1992), and heterosis should, therefore, be taken into account through crossbred information. For example, in our experiment, negative heterosis was observed for both mortality and lesions (Chapter 3). Still pure-line information also remains important, because crossbred sib information alone increases correlations between pure-line full-sib breeding values (i.e. within family deviations cannot be distinguished), thereby reducing accuracy and intensity of selection. Optimally, combined information from crossbreds and pure-line selection candidates should, therefore, be used (Wei and Van der Werf, 1994; Bijma and Van Arendonk, 1998). Whether information should be collected in-ovo, at hatch, later in the growing period in group-housed chicks, or after euthanization will depend on the indicator trait.

Information on clinical traits from challenge testing should be collected from pure-line sibs or progeny as well as crossbreds, because this leads to the highest genetic gain.

Whether challenge testing of pure-line sibs or progeny is done on selection candidate level or multiplier level is more an organizational matter than a matter of potential genetic gain. Crossbreds should be especially produced for challenge testing to keep the generation interval as low as possible, and thereby as high genetic gain as possible. This will also circumvent the practical and economic limitations on the pedigree registration of commercial broilers. Crossbred challenge tests, similar to the already existing specific combining ability tests should, therefore, be set up.

7.5 Additional Considerations

7.5.1 Genotype by Environment Interactions

Genotype by environment interactions may complicate the selection against susceptibility to colibacillosis. Genotype by environment interactions can be indicated by genetic correlations between a given trait in different environments that are less than unity. For example, the correlation between resistance to worms and production traits in merino sheep was small (-0.05) in a low challenge environment, but higher (-0.40) in a moderate challenge environment (Piper and Barger, 1988). In the given example, if selection is done in the low challenge environment then selected animals will perform well in the low challenge environment, but not in the moderate challenge environment. For selection against susceptibility to colibacillosis, there are at least three environments that differ with regard to pathogenic pressure: the pure-line or selection environment; the commercial or breeding goal environment; and the challenge environment where information is collected. Among these environments, genotype by environment interactions may exist. If there is genotype by environment interaction for the susceptibility to colibacillosis then this must be taken into account in the breeding program by ensuring information from an environment that reflects the commercial environment. With increasing genotype by environment interaction, information from the commercial environment, i.e. the breeding goal environment, becomes increasingly important (Mulder and Bijma, 2005).

7.5.2 Maternal Effects

Maternal effects may complicate the selection against susceptibility to colibacillosis as well. Maternal effects are expected to play an important role in susceptibility to colibacillosis, because factors such as the age of the hen and size of the egg are known to have an effect on chick vitality in general. The age of the hen has an influence on the egg quality, e.g. shell strength and integrity; egg contamination and size; and maternal antibodies. The size of the egg has an influence on embryo development and chick hatching weight, possibly through heat production differences and nutrient availability

(Leeson, 2005; Lourens et al., 2006). Further, maternal antibodies, which may play a role in the susceptibility to colibacillosis (Paragraph 7.3.1), would contribute to maternal effects. Several or all of these factors are influenced not only by the maternal genotype, but also by the maternal environment (e.g. nutrition, antigenic exposure, health status). Maternal effects should therefore be taken account of in the selection against susceptibility for colibacillosis, by including them in the estimation of genetic parameters and in genetic evaluations, as it is already done for other traits, such as body weight.

7.5.3 Associative Genetic Effects

The recent developments in group-selection methods, allowing for selection on associative effects, offer a future opportunity for the selection against susceptibility to colibacillosis (Bijma et al., 2007). An associative effect is defined as the effect of an individual on its group members, and is hence an interaction effect (Muir and Cheng, 2004). The breeding value of an individual is composed of the individual's own genetic value along with an associative effect, which is the effect of the individual on other group members (Bijma et al., 2007). In infectious diseases, such as colibacillosis, associative effects may be of importance, because it is possible that not only the susceptibility of the individual is heritable, but also the individuals ability to spread the infection. Further research is required though, before selection on associative effects in the susceptibility to colibacillosis can be implemented.

7.5.4 Pathogen Co-evolution

The genetic gain from selection against susceptibility to colibacillosis may not come to full expression as a consequence of pathogen co-evolution. Selection against susceptibility to colibacillosis is likely to also exert selection pressure on *E. coli*. As a consequence, there is a risk that the net-effect of selection is absent or even negative, because increased host resistance results in increased selection for pathogenicity (Woolhouse et al., 2002). This effect of host resistance on pathogenicity has been shown in vaccination experiments. Vaccines that function by reducing pathogen growth rate and/or toxicity (and not by blocking infection) have been shown to potentially lead to increased pathogenicity (Gandon et al., 2001). Genetic selection for reduced susceptibility to colibacillosis may have the same effect on pathogen evolution as a vaccine would. Therefore, it will be of importance to continuously monitor and explore the developments in the *E. coli* flora in the commercial broiler environment.

7.6 Towards Selection against Susceptibility to Colibacillosis in Practice

A future strategy for selection against susceptibility to colibacillosis has been recommended (Paragraph 7.3.3), where selection is based on clinical traits in response to a semi-natural challenge in an environment with a relatively permanent pathogenic pressure consisting of a cocktail of pathogenic *E. coli*.

Implementation of selection against susceptibility to colibacillosis in practice requires that breeding companies are convinced that colibacillosis is of significant economic importance. The economic importance of colibacillosis depends on disease incidence, production costs and losses associated with sick or dead chicks, as well as market pricing and a non-market economic value, which includes socio-economic aspects, including welfare and food safety concerns of consumers (Nielsen et al., 2005). Colibacillosis is of major economic importance (Vandekerchove et al., 2004; Laughlin, 2005) but to our knowledge, its individual importance has not yet been quantified. For cellulites, which is also caused by *E. coli*, annual losses due to abattoir condemnations in the United States and Brazil have been estimated at $40 and $10 million respectively, and 45.2% of all disapprovals in Brazil are due to cellulites (Laughlin, 2005). Further, septicemia/toxemia and airsacculitis, both highly associated with *E. coli* condemnations, have been identified as the main causes of abattoir condemnations in the United States (Dawe, 2004). For example, in a representative routine survey, they amounted to around 95% of all full condemnations (Collet and Villegas, 2006).

The economic importance of bacterial diseases as a whole is, however, much higher than that of colibacillosis alone, and for many bacterial diseases, such as colibacillosis, it is difficult to argue why selection should be done against that disease rather than another bacterial disease. In the context of an entire breeding program, inclusion of all bacterial diseases in the breeding goal *separately* would make genetic parameter and breeding value estimation very complicated due the large number of traits, and a high number of different challenge tests would be needed to acquire information on all traits separately. The importance of bacterial diseases as a whole should be accounted for in the breeding program though, and a relatively simple solution would be to change the breeding goal to reducing the incidence of bacterial diseases as a whole. An additional advantage of this approach would be a simplified quantification of the economic importance and monitoring of genetic gain, because typing of the bacterial pathogen(s) of a given case would not be necessary. Monitoring of genetic gain can then be organized by keeping records of mortality and veterinary visits along with body weight uniformity at broiler farm level and condemnations at abattoirs. Information on traits for selection should then be acquired through a semi-natural challenge tests with a cocktail of many different pathogens rather than *E. coli*.

There have been reports of selection for specific resistance to one disease resulting in negatively correlated responses in the resistance to other diseases (Biozzi et al. 1979; Thorp and Luiting, 2000). It has, therefore, been suggested that improved health should be achieved through selection on general rather than specific immunocompetence or liveability (Mallard et al., 1998; Wilkie and Mallard, 1999; Knap and Bishop, 2000), unless there is a very large incitement to select against a specific disease. Selection for general immunocompetence may, for example, be done by selection for both innate and adaptive immune system variables (Wilkie and Mallard, 1999; Pinard-van der Laan and Monvoisin, 2000; Kramer et al., 2003), and selection for general liveability may, for example, be done by selection for survival in the commercial environment. Selection against bacterial diseases in general is a modified case of selection for general liveability. Through the use of challenge testing, and supposedly less variability in the biological background of traits measured, it is expected to result in higher genetic gain in the breeding goal. Therefore, selection should be done either against colibacillosis, or if more bacterial diseases are considered of high importance, against bacterial diseases in general.

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Summary

Diseases caused by bacteria in the broiler industry are a main concern in broiler industry, and considerable research emphasis is placed on limiting the impact of these diseases through development of new vaccines and alternative disease control strategies. Bacterial infections are an important welfare issue for the chickens, but they are also economically important. Bacterial diseases of major economic importance include colibacillosis. The ban of the use of all prophylactic antibiotic growth promoters enforced by the EU from the 1st of January 2006, has further stimulated the demand for alternative disease control strategies against bacterial infections. Further, broiler health is not only important for economic and welfare concerns, but also for human food-safety concerns. This dissertation focusses on *E. coli*, which is a part of the normal flora in chickens, because this is the pathogen with highest importance for both broiler health and human food-safety. Several important diseases in broilers, including respiratory colibacillosis, are associated with *E. coli*. Mortality due to respiratory colibacillosis usually stays below 5% but the proportion of disease cases often reaches more than 50%. In broilers, the prevalence of colibacillosis is lower in the first half of the production period, where it occurs as a primary infection, than in the second half, where it predominantly occurs as a secondary infection with potential primers ranging from environmental factors to viral infections or vaccinations against these viruses. Traditional management strategies, such as vaccinations and preventive use of as well as therapeutic treatment with antibiotics, are insufficient in preventing colibacillosis, and genetic selection may offer an eligible supplementive preventive strategy. There are indications that genetic variation exists between chicks in susceptibility and hence that genetic selection to reduce susceptibility to colibacillosis is possible.

This dissertation basically aimed to evaluate broiler susceptibility to colibacillosis and the potential of genetic selection to reduce broiler susceptibility to colibacillosis.

The evaluation of the possible presence of a genetic component in the susceptibility to colibacillosis is complicated, because, in literature, no clear and consistent definition of the susceptibility to colibacillosis has been given and differing evaluation criteria have been used. **Chapter 2** aimed to define the susceptibility of broilers to colibacillosis through quantification of clinical responses and to examine the relationship between susceptibility and growth retardation. A challenge experiment was carried out twice. In each trial, 192 chicks were challenged intratracheally with *E. coli* at 7 days of age and 160 chicks served as controls. Surviving chicks were euthanized at 14 or 15 days. Traits measured were: daily mortality, lesion scores (airsacculitis, pericarditis, and

perihepatitis), body weight at 1, 4, 7, 10, 12 and 14 or 15 days and feeding behaviour at 6, 11 and 13 days. The results were reproducible, and increasing susceptibility to colibacillosis was defined by four categories: chicks without lesions, chicks with airsacculitis but no systemic lesions, chicks with systemic lesions, and chicks that die. Increasing susceptibility was associated with increasing growth retardation, but growth retardation was not inevitably linked to challenge with *E. coli*.

Selection for reduced susceptibility to colibacillosis in broilers may contribute to the prevention of colibacillosis. Such selection should focus on the responses to *E. coli* rather than the associated primary agent(s). The above-described challenge experiment included chicks from five pure broiler lines, a slow-growing line, and two 2-way crosses of the pure-lines (altogether referred to as genotypes). Per trial, 24 chicks per genotype were challenged and 20 chicks per genotype were controls. **Chapter 3** aimed to examine whether genetic variation is present in the susceptibility to colibacillosis through an evaluation of the susceptibility to primary colibacillosis in the eight genotypes. An effect of genotype on mortality, lesion prevalence, and growth retardation was found, indicating the presence of genetic variation in susceptibility to colibacillosis, and suggesting that selection for reduced susceptibility is possible. There were large between-genotype differences in mortality (up to 46%) and in lesion prevalence (up to 41%). Growth retardation was not observed for any genotype in chicks without lesions, whereas genotypes differed from none to 20% growth retardation for chicks with airsacculitis but no systemic lesions, and up to 13% for chicks with systemic lesions. The heterosis in susceptibility and growth retardation was found to be either negative or absent, indicating that crossbreeding would not be an advantage for the selection for reduced susceptibility, and that test crossing is essential.

The biological background of the variation in susceptibility to colibacillosis is not yet fully understood, although this is essential to foresee contingent negative side effects of selection. **Chapter 4** basically had two aims: 1) investigating whether differences in susceptibility to colibacillosis are associated with maternal antibodies, antibody response, and alterations in thyroid hormones [triiodothyronine (T_3) and thyroxine (T_4)]; and 2) investigating the effect of genotype on the changes in T_3 and T_4 during challenge and antibody response. In the above-described challenge experiment thyroid hormone plasma concentrations and *E. coli* specific antibody titers (*AB*) were measured at 7 d (T_{3d7}, T_{4d7} , and AB_{d7}) and 14 or 15 d (change from 7 to 14 or 15 d was analyzed: ΔT_3, ΔT_4, and ΔAB). There was a significant effect of challenge on T_{3d7}; probably due to eating pattern in association with circadian rhythm. The challenge group was suggested

to have functional hypothyroidism relative to the control group, indicating metabolic changes due to the challenge, and it was indicated that an antibody response was elicited. Differences in susceptibility were not significantly related to differences in T_{3d7}, T_{4d7}, ΔT_3, or ΔT_4 or to maternal antibodies (AB_{d7}), but the antibody response tended to increase (decreasing ΔAB) with increasing susceptibility. There were indications of genetic variation in T_{4d7}, ΔT_4, AB_{d7}, and ΔAB, but there was no observed effect of genotype on ΔT_3 and ΔT_4 during challenge or on the antibody response. Further, there were indications that selection for growth traits has resulted in alterations in ΔT_4 due to challenge, as indicated by a lower ΔT_4 in the challenge group relative to the control group for more intensively selected genotypes as opposed to a higher ΔT_4 for less intensively selected genotypes.

Difficulties in evaluating immunological variables hinder attempts to improve animal health through selection on immunological variables. In young chicks, evaluating immunological variables is additionally complicated by immune system development and maternal immunity. The evaluation of immunocompetence and immunoresponsiveness, and the definition of appropriate challenge and measurement strategies, may be enabled through a mathematical model which captures the key components of the immune system and its development. **Chapter 5** basically had two aims: 1) to develop a deterministic model that describes immunocompetence development and the kinetics of immunoresponsiveness to a pathogenic challenge in chicks; and 2) to use this model to illustrate the importance of factors in experimental design, such as type of variable measured, measurement timing, and challenge age. A model was developed that describes immunocompetence development as well as kinetics of immunoresponsiveness to a pathogenic challenge in an individual chick from 0 to 56 days of age. The model consisted of four components describing immunocompetence (maternal- and baseline immunity) and immunoresponsiveness (acute phase- and antibody response). Individual component equations generally fitted published data adequatelly. Four scenarios that represented combinations of challenge age and measurement timing were simulated. In each scenario, the immunoresponsiveness to a particular challenge was compared for three different levels of baseline immunity, representing three broiler genotypes. It was illustrated that experimental design (type of immunoresponsiveness measured, measurement timing, and challenge age) can have an important effect on the ranking of genotypes, groups, or individuals, and on the reliability of extrapolations based on this ranking. It is concluded that this model is a potentially useful tool in the definition of appropriate challenge and measurement strategies when evaluating immunocompetence and immunoresponsiveness. Further, it

may be used as a generator of hypotheses on global immunological relationships to be tested experimentally.

The considerable amount of variability in immunological variables may cause non-normalities and heteroscedasticity in variance across age. Accounting for this variability in evaluation of challenge and measurement strategies for selection may be enabled through a stochastic model of immunocompetence development and immunoresponsiveness kinetics. **Chapter 6** basically had two aims: 1) to develop a stochastic model that reflects observed variation between animals and across age in immunocompetence and responsiveness; and 2) to illustrate consequences of this variability for statistical power of genotype comparisons and selection. A stochastic model was developed by expanding the model described in Chapter 5 to a population of individual chicks with variation among them as well as stochastic variation within individuals across age. The variation in the model reflected variability observed in literature in some cases. In some studies, however, variability varied randomly across age, possibly caused by small sample sizes or non-normalities, as skewness in immunological variables is common. Due to heteroscedasticity in variance, expected statistical power of experiments may not be achieved in practice. This was also illustrated with the model. The model predicts that heteroscedasticity in variance across age decreases with increasing challenge age. Therefore, a high challenge age, at which maternal immunity no longer has influence, is preferable. Causal relationships within and among immunocompetence and responsiveness components in the model resulted in correlations among them and within them across age. These correlations were generally similar to those in literature. Due to low repeatabilities of immunocompetence and immunoresponsiveness across age, selection for improved immuno-competence or responsiveness at a given age may not result in the desired response at other ages. Indeed, the model predicts that the average and minimum probability to detect a given difference at another age than the selection age increases with increasing selection age. Therefore, the age at selection, and the age at which information is gathered for selection, must be considered carefully regarding the age at which the immunocompetence or responsiveness is desired to be improved.

In **Chapter 7**, the experimental design used in Chapters 2, 3, and 4, the main findings of this dissertation, and the most important hypotheses generated were evaluated and discussed in relation to the potential of genetic selection to reduce broiler susceptibility to colibacillosis. In a selection strategy context, evaluation of susceptibility to colibacillosis should always be done within each line or crossbred of interest, and

potentially low genetic correlations between susceptibility to colibacillosis at "young" and "older" age must be taken into account. In future genetic evaluations, information should be gathered on individual feed intake, daily mortality, the presence of airsacculitis and systemic lesions, as well as daily body weight measurements in challenge tests. Challenge testing should be based on a semi-natural challenge with a cocktail of pathogenic *E. coli*, and before beginning actual genetic evaluations, expected within-line mortality resulting from challenge testing should be estimated to ensure sufficient information on other traits for selection.

The breeding goal for selection against susceptibility to colibacillosis should be to reduce the incidence of colibacillosis at commercial broiler level, and if susceptibility to colibacillosis is not the same trait at different ages then the breeding goal should be to reduce the incidence of colibacillosis at "older" ages only. Selection against susceptibility to colibacillosis should be done by direct selection on clinical traits until indicator traits have been verified, whereafter selection should be based on a combination of the forces of indicator and clinical traits. Promising indicator traits are organ weights, especially bursa of fabricius, and ciliary activity, but also natural antibodies in the respiratory tract may be promising. Important clinical traits are survival, organ weights, and growth retardation. Indicator traits should be measured directly on selection candidates, as well as on challenge tested animals. Information on clinical traits from challenge testing should be collected from pure-line sibs and progeny as well as crossbreds, especially produced for challenge testing.

Additional considerations for setting up a breeding program, includes genotype by environment interactions, maternal effects, and pathogenic co-evolution. If there is genotype by environment interaction for the susceptibility to colibacillosis then this must be taken into account in the breeding program. This can be done by collecting information from an environment, which resembles the breeding goal environment as much as possible, on crossbred progeny, and by not putting emphasis on information collected in the challenge environment for the genetic evaluation of production traits. Maternal effects should be taken account of in the selection against susceptibility for colibacillosis, by including them in the estimation of genetic parameters and in genetic evaluations. Pathogenic co-evolution should continuously be monitored and explored, because it may be a cause of contingently lower genetic gain than expected.

Finally, the economic importance of bacterial diseases as a whole is much higher than that of colibacillosis alone, and changing the breeding goal to reducing the incidence of bacterial diseases as a whole is, therefore, sensible.

Samenvatting (Dutch Summary)

Ziekten die veroorzaakt worden door bacteria in de vleeskuiken industrie zijn een grote zorg voor de vleeskuiken industrie. Er wordt aanzienlijke nadrukkelijk aandacht gegeven aan onderzoek naar mogelijkheiden om door middel van het ontwikkelen van nieuwe vaccines en alternatieve strategiën om deze ziektes te bestrijden. Bacteriele infecties hebben een belangrijke invloed op het welzijn van de kuikens, maar ze zijn ook economisch gezien belangrijk. Tot bacteriële ziekten met groot economisch belang hoort ook colibacillose. Het verbod op het gebruik van alle prophylactische antibiotische groei bevorderaars die is opgelegd door de EU vanaf 1 januari, 2006, heeft de vraag naar alternatieve ziektebestrijdingstrategieën tegen bacteriële infecties verder gestimuleerd. Vleeskuikengezondheid is bovendien niet alleen belangrijk voor economie en welzijn, maar ook voor de humane voedselveiligheid. Dit proefschrift richt zich op *Escherichia coli* (*E. coli*), die deel uitmaakt van de normale flora van kuikens. Dit pathogeen speelt de belangrijkste rol in zowel vleeskuikengezondheid als humane voedselveiligheid. Meerdere belangrijke ziekten in vleeskuikens, waaronder respiratoire colibacillose, zijn geassocieerd met *E. coli*. Uitval vanwege respiratorische colibacillose blijft meestal onder de 5%, maar de proportie ziektegevallen komt vaak boven de 50%. In vleeskuikens is de prevalentie van colibacillose lager in de eerste helft van de productieperiode dan in de tweede helft. In de eerst helft komt het voor als een primaire infectie, in de tweede helft voornamelijk als een secondaire infectie met potentiële triggers die variëren van milieufactoren tot virale infecties of vaccinaties tegen deze virussen. Traditionele beheerstrategieën, zoals vaccinaties en preventieve therapeutische behandeling met antibiotica, zijn onvoldoende om colibacillose te voorkomen. Genetische selectie is een mogelijke aanvullende strategie op preventie. Er zijn indicaties dat genetische variatie bestaat in gevoeligheid tussen kuikens en daarmee dat genetische selectie voor het beperken van gevoeligheid voor colibacillose mogelijk is.

Dit proefschrift heeft tot doel om te onderzoeken hoe gevoelig vleeskuikens zijn voor colibacillose en wat de mogelijkheden zijn om de gevoeligheid van vleeskuikens voor colibacillose door selectie te beperken.

De evaluatie van het mogelijke bestaan van een genetische component in de gevoeligheid voor colibacillose is gecompliceerd, omdat er in de literatuur geen duidelijke en consistente definitie is gegeven en verschillende evaluatie criteria zijn gebruikt. **Hoofdstuk 2** had tot doel om de gevoeligheid van vleeskuikens voor colibacillose te definiëren door middel van kwantificering van klinische reacties en om de relatie tussen gevoeligheid en groei vertraging te onderzoeken. Een challenge

experiment werd uitgevoerd in tweevoud. In beide tests werd 192 kuikens intratracheaal gechallenged met *E. coli* op een leeftijd van 7 dagen en 160 kuikens dienden als controle. Overlevende kuikens werden euthanaseert op een leeftijd van 14 of 15 dagen. Kenmerken die gemeten werden tijdens het experiment zijn: uitval, laesie score (luchtzak ontsteking, pericarditis en perihepatitis), lichaamsgewicht op 1, 4, 7, 10, 12 en 14 of 15 dagen en eetgedrag op 6, 11 en 13 dagen. De resultaten waren reproduceerbaar en toenemende gevoeligheid voor colibacillose werd gedefinieerd in vier categorieën: kuikens zonder laesies, kuikens met luchtzakontsteking maar geen systemische laesies, kuikens met systemische laesies, en kuikens die doodgingen. Toenemende gevoeligheid was geassocieerd met toenemende groei vertraging, maar groei vertraging was niet noodzakelijkerwijs verbonden met *E. coli* challenge.

Selectie voor verminderen van gevoeligheid voor colibacillose in vleeskuikens zou een mogelijke bijdrage kunnen leveren aan de preventie van colibacillose. Dergelijke selectie zou zich moeten richten op de reacties op *Escherichia coli* besmetting en niet op de geassocieerde trigger(s). Kuikens van vijf verschillende zuivere vleeskuiken lijnen, een langzaam groeiende lijn, en twee twee-weg kruisingen (in totaal genotypen genoemd) maakten deel uit van de bovengenoemde challenge experiment. Per test werden 25 kuikens per genotype gechallenged en 20 kuikens per genotype dienden als controle. **Hoofdstuk 3** had tot doel om te onderzoeken of er genetische variatie bestaat in de gevoeligheid voor colibacillose door deze gevoeligheid in de acht genotypen te evalueren. Een effect van genotype was gevonden voor uitval, laesie prevalentie en groei vertraging, wat duidt op het bestaan van genetische variatie in gevoeligheid voor colibacillose en suggereert dat selectie voor verminderde gevoeligheid mogelijk is. Er waren grote verschillen tussen genotypen in uitval (tot 46%) en in laesie prevalentie (tot 41%). In geen van de genotypen lieten kuikens die geen laesies hadden groeivertraging zien. De genotypen verschilden van 0% tot 20% groeivertraging in de kuikens die luchtzak ontsteking maar geen systemische laesies hadden en tot 13% in groeivertraging in de kuikens die systemische laesies hadden. De heterosis voor gevoeligheid en groeivertraging was negatief of afwezig wat erop duidt dat crossbreeding niet voordelig zou zijn bij selectie voor verminderde gevoeligheid en dat testkruisingen essentieel zijn.

De biologische achtergrond van de variatie in gevoeligheid voor colibacillose is nog niet volledig duidelijk, hoewel dit wel essentieel is om mogelijke negatieve neveneffecten van selectie te voorzien. **Hoofdstuk 4** had twee doelen: 1) om te onderzoeken of verschillen in gevoeligheid voor colibacillose is geassocieerd met maternale antilichamen, antlichaam respons en veranderingen in schildklierhormonen

[triiodothyronine (T_3) en thyroxine (T_4)]; en 2) om het effect van genotype op de veranderingen in T_3 and T_4 tijdens challenge en op antilichaam respons te onderzoeken. In het bovengenoemde challenge experiment, werden schildklierhormoon-concentraties in het plasma en *E. coli* specifieke antilichaam titers (*AB*) gemeten op 7 dagen (T_{3d7}, T_{4d7} en AB_{d7}) en op 14 of 15 dagen (de verschillen tussen 7 en 14 of 15 dagen werden geanalyseerd: ΔT_3, ΔT_4 en ΔAB). Er was een significant effect van challenge op T_{3d7}; waarschijnlijk vanwege eetpatronen gerelateerd aan het dagelijkse ritme. Het werd gesuggereerd dat de challenge groep functionele hypothyroidism relatief tot de controle groep had, wat duidt op metabolische veranderingen vanwege de challenge, en het werd uitgelegd als dat een antilichaam respons werd gegeven. Verschillen in gevoeligheid waren noch significant gerelateerd tot verschillen in T_{3d7}, T_{4d7}, ΔT_3 of ΔT_4, noch in maternale antilichamen (AB_{d7}), maar er was een tendens tot een toename in antilichaam respons (afname in AB_{d7}) met toenemende gevoeligheid. Er waren aanwijzingen voor genetische variatie in T_{4d7}, ΔT_4, AB_{d7} en ΔAB, maar er was noch een significant effect van genotype op ΔT_3 en ΔT_4 tijdens challenge noch op de antilichaam respons. Er waren verder aanwijzingen dat selectie voor groei kenmerken heeft geresulteerd in veranderingen in ΔT_4 vanwege challenge. Dit bleek uit een lagere ΔT_4 in de challenge groep, relatief tot de controle groep voor genotypen die intensiever geselecteerd zijn in tegenstelling tot een hogere ΔT_4 voor genotypen die minder intens geselecteerd zijn.

Moeilijkheden in het evalueren van immunologische parameters belemmeren pogingen om diergezondheid te verbeteren door middel van selectie. In jonge kuikens is het evalueren van immunologische parameters verder gecompliceerd door de ontwikkeling van het immuunsysteem en maternale immuniteit. Het evalueren van immunocompetentie en immunoreactiviteit en het definiëren van geschikte challenge en meetstrategiën, kan mogelijk bereikt worden met een wiskundig model die de belangrijkste componenten van het immuunsysteem en hun ontwikkeling beschrijft. **Hoofdstuk 5** had twee doelen: 1) om een deterministisch model te ontwikkelen die de ontwikkeling van immunocompetentie en kinetiek van immunoreactiviteit tegen een pathogeen challenge in kuikens beschrijft; en 2) om dit model te gebruiken om het belang van factoren, zoals het type variabele dat gemeten wordt, de timing van metingen en challenge leeftijd, in experimentele opzet te illustreren. Een model dat de ontwikkeling van immunocompetentie en kinetiek van immunoreactiviteit tegen een challenge met een pathogeen in een individueel kuiken van 0 tot 56 dagen beschrijft werd ontwikkeld. Het model bestond uit vier componenten die immunocompetentie (maternale en basale immuniteit) en immunoreactiviteit (acute phase en antilichaam respons). De componenten kwamen goed overeen met gepubliceerde data. Vier

scenarios die verschillende combinaties van challenge leeftijd en timing van metingen weergaven werden gesimuleerd. In elk scenario werd de immunoreactiviteit tegen een bepaalde challenge vergeleken voor drie verschillende niveaus van basale immuniteit die drie vleeskuiken genotypen moesten weergeven. Het werd geïllustreerd dat type van immunoreactiviteit gemeten, timing van metingen en challengeleeftijd een belangrijk effect kunnen hebben op de rangschikking van genotypen, groepen of individuen en op de betrouwbaarheid van extrapolaties die op deze rangschikking gebaseerd zijn. Conclusie was dat dit model een nuttig instrument is voor het definiëren van geschikte challenge en meet strategieën wanneer immunocompetentie en immunoreactiviteit worden geëvalueerd, en dat het model gebruikt kan worden om hypothesen te genereren over globale immunologische verhoudingen die experimenteel getoetst kunnen worden.

De aanzienlijke hoeveelheid variabiliteit in immunologische variabelen kan non-normaliteit en heteroscedasticiteit in variantie over leeftijden heen veroorzaken. Door middel van een stochastisch model voor immunocompetentie ontwikkeling en immunoreactiviteit kinetiek kan er rekening gehouden worden met deze variabiliteit in de evaluatie van challenge en meetstrategieën voor selectie. **Hoofdstuk 6** had twee doelen: 1) om een stochastisch model te ontwikkelen dat waargenomen variatie tussen dieren en over leeftijden heen in immunocompetentie en reactiviteit modelleert; en 2) om de gevolgen van deze variatie op de statistische power van genotype vergelijkingen en selectie te illustreren. Een stochastisch model werd ontwikkeld door het model, dat in Hoofdstuk 5 werd beschreven, uit te breiden door variatie tussen individuen en binnen individuen over leeftijden heen toe te laten. De variatie in het model geeft in sommige gevallen de variatie beschreven in de literatuur goed weer. In sommige studies varieerde de variabiliteit echter over leeftijden heen, mogelijk veroorzaakt door kleine sample grootte of non-normaliteiten daar skewness normaal is in immunologische variabelen. Vanwege heteroscedasticiteit in variantie wordt in de praktijk de verwachtte statistische power van experimenten niet altijd behaald. Dit werd ook geïllustreerd met het model. Het model voorspelt dat heteroscedasticiteit in variantie over leeftijden heen afneemt met toenemende challengeleeftijd. Daarom is een hogere challengeleeftijd, waarbij de maternale immuniteit niet langer van invloed is, te prefereren in een experiment. Er waren correlaties binnen (over leeftijden heen) en tussen immunocompetentie en reactiviteitcomponenten in het model als gevolg van causale relaties binnen en tussen de componenten. Deze correlaties stemden over het algemeen overeen met die in de literatuur. Selectie voor verbeterde immunocompetentie of immunoreactiviteit op een bepaalde leeftijd resulteert niet per-se in de gewenste respons op andere leeftijden, mede door lage herhaalbaarheden van immunocompetentie en immunoreactiviteit over

leeftijden heen. Het model voorspelt dat de gemiddelde en minimum waarschijnlijkheid om een gegeven verschil op een andere leeftijd dan de leeftijd van selectie waar te nemen toeneemt met een toenemende leeftijd van selectie. De leeftijd van selectie, zowel als de leeftijd waarop informatie is verzameld voor selectie, moeten daarom zorgvuldig in aanmerking genomen worden met betrekking tot de leeftijd waarop het gewenst is om de immunocompetentie of reactiviteit te verbeteren.

Het experimentele design dat gebruikt werd in Hoofdstukken 2, 3 en 4, de voornaamste bevindingen van dit proefschrift, en de belangrijkste hypothesen die gegenereerd zijn werden in **Hoofdstuk 7**, geëvalueerd en bediscussieerd in relatie tot de mogelijkheid van genetische selectie tegen vleeskuikengevoeligheid voor colibacillose. In de context van selectiestrategieën moet de evaluatie van gevoeligheid voor colibacillose altijd binnen de lijn of kruising uitgevoerd worden en er moet rekening gehouden worden met potentieel lage genetische correlaties tussen gevoeligheid voor colibacillose op "jonge" en "oudere" leeftijd. In toekomstige genetische evaluaties moet informatie op individuele voeropname, dagelijkse uitval, de aanwezigheid van luchtzakontsteking en systemische laesies en dagelijkse lichaamsgewichten verzameld worden in challengetests. Challengetesten moeten gebaseerd worden op een semi-natuurlijke challenge met een cocktail van pathogene *E. coli*, en voor het opstarten van feitelijke genetische evaluaties, de verwachte uitval binnen lijnen in de challengetesten moet geschat worden om te zorgen dat voldoende informatie op andere kenmerken voor selectie verkregen kan worden.

Het fokdoel voor selectie tegen gevoeligheid voor colibacillose zou verlaging van de incidentie van colibacillose op commercieel vleeskuiken moeten omvatten. Als gevoeligheid voor colibacillose op verschillende leeftijden niet hetzelfde kenmerk blijkt te zijn, zou het alleen de incidentie van colibacillose te verlagen op "oudere" leeftijden moeten omvatten. Totdat indicator kenmerken geverifieerd zijn moet selectie tegen gevoeligheid voor colibacillose gedaan worden op basis van directe selectie op klinische kenmerken. Hierna moet selectie gedaan worden op basis van een combinatie van indicator en klinische kenmerken. Veelbelovende indicator kenmerken zijn orgaangewichten, vooral de bursa, en activiteit van respiratorische trilharen, maar ook natuurlijke antilichamen in de luchtwegen zou veelbelovend kunnen zijn. Belangrijke klinische kenmerken zijn overleving, orgaangewichten en groeivertraging. Indicator kenmerken moeten direct op selectie kandidaten gemeten worden, zowel als op dieren die gechallenged zijn. Informatie op klinische kenmerken van challengetests moet verzameld worden van zussen en broers van zuivere lijnen en van kruisingen die speciaal gemaakt zijn voor het challengetesten.

Bijkomende afwegingen voor het opzetten van een fokprogramma omvatten genotype-milieu interacties, maternale effecten en pathogeen co-evolutie. Als er genotype-milieu interacties zijn voor de gevoeligheid voor colibacillose, dan moet er rekening mee gehouden worden in de fokprogramma. Dit kan gedaan worden door het verzamelen van informatie van kruisingsnakomelingen in een milieu dat zoveel mogelijk op het praktijkmilieu lijkt en door niet nadruk te leggen op informatie dat verzameld is in het challengemilieu voor de genetische evaluatue van productie kenmerken. Er moet rekening gehouden worden met maternale effecten in de selectie tegen gevoeligheid voor colibacillose door ze mee te nemen bij het schatten van genetische parameters en in genetische evaluaties. Pathogene co-evolutie moet voortdurend gemonitored en verkend worden, omdat het een oorzaak kan zijn voor een eventueel lagere genetische vooruitgang dan verwacht.

Ten slotte, het economische belang van bacteriële ziektes als geheel is veel groter dan dat van colibacillose alleen, en het is daarom zinvol om het fokdoel te veranderen in het verlagen van de incidentie van bacteriële ziektes als geheel.

Glossary

Acute phase	- protein:	A class of proteins that are synthetized in the liver in response to inflammation.
	- response:	Physiologic changes that occur shortly after the onset of an infection and include an increase in the blood level of various proteins, fever, and other metabolic changes.
Airsacculitis:	Inflammation of the mucous membrane of the air sacs of birds.	
Anorexia:	Loss of appetite, especially as a result of disease.	
Antibodies	- maternal:	Transferred passively from mother to offspring (through the yolk)
	- natural:	Produced independent of internal or external antigenic stimuli with a broad antigen specificity.
Ascites:	An excessive amount of serous fluid in the abdominal cavity.	
Barrier	- biological:	E.g. competition with normal flora, antibiosis, generation of anaerobic or acidic conditions.
	- chemical:	E.g. lysozymes, proteolytic enzymes, fatty acids.
	- physical:	E.g. desiccation, pH, desquamation, mucus, and cilia.
Breeding	- goal:	The breeding goal is the formulation of the desired direction of selection and can include multiple traits.
	- program:	The system in which information on performance of potential breeding animals is gathered and used to estimate breeding values, and superior animals are selected and used to breed the next generation.
	- value:	The value of an individual, judged by the mean value of its progeny for a given trait or traits.
Cell	- mediated immune response:	The immune response produced when sensitized T cells attack foreign antigens and secrete lymphokines that initiate the body's humoral immune response.
Cellulitis:	Inflammation of connective tissue.	
Central Limit Theorem:	If the sum of the variables has a finite variance, it will be approximately normally distributed.	
Coli	-bacillosis (primary / secondary):	Infections in poultry caused by *Escherichia coli* leading to various disease conditions such as coligranuloma, perihepatitis, pericarditis, and air sac disease.
	-granuloma:	Also called Hjarre's disease. A condition in adult fowl characterized by granulomatous lesions in the liver, cecum, spleen, bone marrow, and lungs.
Commercial	A broiler at commercial level that is produced for meat production. Usually	

broiler:	a three- or four-way cross and four generations from the pure-lines that it descents from.	
Complement system:	A complex series of proteins that interact with antigen-antibody complexes that are involved in immune and inflammatory reactions.	
Correlated response:	A change in a given trait, A, due to selection on another trait, B.	
Crossbred:	A hybrid offspring of parents from two genetically distinct populations.	
Cytokine:	Regulatory proteins, such as the interleukins and lymphokines, that are released by cells of the immune system and act as intercellular mediators in the generation of an immune response.	
Epistatic effect:	Epistatic interaction is the interaction between genes.	
Fibrinous exudate:	A generally viscous, yellowish-white fluid formed in infected tissue, consisting of white blood cells, cellular debris, and necrotic tissue.	
Fitness trait:	A trait of importance for fitness, where fitness is defined as the contribution of offspring to the next generation.	
Fowl cholera:	An acute diarrheal disease (especially in chickens) caused by the microorganism that causes hemorrhagic septicaemia.	
Generation interval:	The average age of the parents at the birth of their selected offspring.	
Genetic	- correlation:	Covariation of features in populations because they share some genes. An association between the genes that determine two traits.
	- evaluation:	The evaluation of individuals in a population based on their genetic (breeding) value.
	- gain:	The average (heritable) change from one generation to the next as a result of selection.
	- parameter:	Genetic variance, correlation or heritability
	- variation (additive):	Genetic variance that arises from the additive effects of genes on the phenotype.
Genotype:		A group of individuals sharing a specific genetic constitution.
	- by environment interaction:	Differences in environmental deviations depending on genotype. Due to genotype by environment interactions, the genetic ranking of individuals may differ among environments.
Gram-negative:	Bacteria that have a thick double cell wall that contains lipopolysaccharide or endotoxin; these bacteria lose a violet stain when rinsed.	

Growth retardation:	Reduced growth relative to the norm.	
Heritability:	The proportion of the total phenotypic variance in a trait that is due to the additive effects of genes (narrow sense).	
Heterosce-dasticity in variance:	A condition where the variances of a given trait, A, are not equal for every value of a given factor B.	
Heterosis:	The difference between the cross and the mean of the 2 parent lines.	
Homologous protection:	Vaccines that provide protection against homologous antigens, i.e. antigens that are corresponding or alike in structure, position, or origin, usually resulting from common ancenstry.	
Humoral immune response:	An immune response that is mediated by B cells.	
Immunologic al memory:	The ability to resist reinfection.	
Infection	- secondary bacterial:	Any bacterial infection that follows a primary infection.
	- horizontal:	Infection between individuals in a flock or between flocks.
	- respiratory:	Any infection that infects the respiratory tract.
	- systemic:	Any infection that has infected some part of an organism through the cardiovascular system.
Information source:	The type of individuals on which information on the traits for selection is collected, usually signified by their relatedness with the selection candidates, e.g. full-sib, half-sib, progeny.	
Innate immune response:	A phylogenetically ancient defence mechanism, which uses germline encoded receptors for the recognition of microbial pathogens. The first line of defence.	
Inoculum:	A medium containing organisms, usually bacteria or a virus, that is introduced into cultures or living organisms.	
Ionophores:	Any of a group of organic compounds that form a complex with an ion and transport it across a membrane.	
Isolator:	A test unit in which the environment can be controlled completely, and which is isolated completely from the surroundings.	
Lesions:	Almost any abnormality involving any tissue or organ due to any disease or any injury. In this dissertation, the term lesion is used as short for gross lesions.	

Lesions:	- gross:	A lesion that can be seen with the naked eye. In this dissertation, gross lesions are observations of fibrinous exudate, indicating either airsacculitis, pericarditis, perihepatitis, or all of these.
	- systemic:	In this dissertation, either pericarditis, perihepatitis, or both of these.
Leucosis:	A disease complex in fowl probably caused by viruses and characterized by autonomous proliferation of blood-forming cells.	
Line	- dam:	A pure-line, in which emphasis is less on production traits than in a sire line and more on reproduction traits.
	- pure:	A genetic line (group of individuals related by descent from common ancestors), which is owned by the breeding company and subjected to the full-scale selection program.
	- sire:	A pure-line, in which emphasis is on production traits, i.e. body weight and feed conversion.
	- slow-growing:	A meat-type chicken, which has a lower growth potential than a commercial broiler.
Macrophage:	A type of white blood cell that surrounds and kills microorganisms, removes dead cells, and stimulates the action of other immune system cells. Any mononuclear, actively phagocytic cell arising from monocytic stem cells in the bone marrow.	
Major histocompatibility complex (MHC):	A group of genes that control aspects of the immune response. The products of these genes are present on every cell of the body and serve as markers to distinguish self from nonself cells.	
Marek's disease:	A herpesvirus that causes a lymphoproliferative disease in chickens.	
Maternal effect:	The effect of the maternal genotype on the phenotype of the offspring, or the zygotes.	
Model	- computational:	A mathematical model to study the behavior of a complex system by computer simulation.
	- deterministic:	Every set of variable states is uniquely determined by parameters in the model and by sets of previous states of these variables. Performs the same way for a given set of initial conditions.
	- dynamic:	Accounts for the element of time. Typically represented with difference or differential equations.
	-epidemiological:	Mathematically simulates the progress of an infectious disease in a population.
Multiplier level:	A stage in the breeding pyramid in which the number of chicks is multiplied to increase the reproductive capacity and finally the number of broilers produced.	

Omphalitis:		Inflammation of the umbilicus (the navel).
Osteomyelitis:		Inflammation of the bone marrow and adjacent bone.
Panophthalmitis:		Inflammation of all the structures or tissues of the eye.
Pedigree:		Lineage: the ancestors and descendant of an individual.
Peri	-carditis:	Inflammation of the pericardium-sac enclosing the heart.
	-hepatitis:	Inflammation of the peritoneum and tissues surrounding the liver.
	-tonitis:	Inflammation of the peritoneum, the lining of the abdominal cavity.
Phagocytosis:		The ingestion of micro-organisms, cells, and foreign particles by phagocytes, eg phagocytic macrophages.
Phenotypic variation:		The variation between individuals that is observable, i.e. it includes both genetic and environmental variation.
Polyserositis (fibrinous):		Chronic inflammation of several serous membranes with effusions in serous cavities resulting in fibrous thickening of the serosa and constrictive pericarditis.
Power	- statistical:	The power of a statistical test is the probability that the test will reject a false null hypothesis, or in other words that it will not make a Type II error.
Primer:		Any substance or pathogen, which is necessary for the secondary infection.
QTL:		Quantitative trait locus. A region of DNA that is associated with a particular trait. Though not necessarily genes themselves, QTLs are stretches of DNA that are closely linked to the genes that underlie the trait in question.
Repeatability:		Repeatability is in this dissertation a measure of the correlation of a given trait within an individual, across age.
Salmonellosis:		Also called Pullorum disease. Infection in chickens with bacteria of the genus *Salmonella*.
Salpingitis:		Inflammation of fallopian tube (part of the female reproductive tract. There are two long slender fallopian tubes through which eggs pass from the ovaries to the uterus.).

Selection		The act of choosing favourable offspring as parents for future generations based on their genetic value.
	- candidate:	An individual that potentially can be selected to produce the next generation.
	- criterium (direct / indirect):	A trait based on which a genetic evaluation of selection candidates is done and based on which selection decisions are finally done. A direct selection criterium is a trait, which provides direct information on the breeding goal. An indirect selection criterium is a trait, which provides indirect information on the breeding goal.
	- divergent:	The selection of a genotype in two directions, opposite of each other.
	- group:	Selection for traits that would be beneficial to a population at the expense of the individual possessing the trait.
	- response (direct / indirect):	The change of the population mean produced by selection. Direct selection: selection is performed on the same trait as the trait in which a response is desired. Indirect selection: selection is performed on one trait to obtain a correlated response in another trait.
	- strategy:	The strategy of selection: decisions on breeding goal, traits to measure, and information sources.
Septicaemia (acute):		Blood poisoning. Rapid multiplication of bacteria and toxin production within the blood.
Serotype:		A subdivision of a species of microorganism, eg, a bacteria, based upon its particular antigens.
Stock:	- great-grand parent:	The progeny of the pure-lines. Subjected to limited (usually mass) selection for selected traits. This generation is mainly used to multiply the pure-lines to to produce the grandparent stock.
	- grand parent:	The progeny of the great-grandparent stock. This is generally the first generation in which crossbreeding is performed.
	- parent:	The progeny of the grandparent stock. This is generally the second generation in which crossbreeding is performed.
Swollen head syndrome:		A disease of chickens caused by the turkey rhinotracheitis virus.
Synovitis:		Inflammation of the synovium (a thin layer of connective tissue with a free smooth surface that lines the capsule of a joint).
Uniformity:		The percentage of individuals with a body weight between mean body weight minus 10% and mean body weight plus 10%.

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Birgitte ☺

Curriculum Vitae

Birgitte Ask was born on the 27th of December 1975 in Denmark. In 1994, she graduated from Gymnasium (DK), and in that same year, she joined the Australian Trust for Conservation Volunteers and travelled round Australia. Later she attended the first year of Nature Sciences at Odense University (DK) until she started studying 'Agronomy' at the Royal Veterinary and Agricultural University (KVL) in Copenhagen (DK) in 1996. During her Masters in Animal Science, she visited Wageningen University (NL). First as an ERASMUS student in 1999-2000, and later on an ad hoc basis from 2001 until she finished her studies. She obtained the degree of Cand. Agro. from KVL, and MSc in Animal Science with specialisation in Animal Breeding and Genetics from Wageningen University, in 2002 and 2003 respectively. During her studies she also did an internship at the broiler breeding company, Hybro B.V. Since 2002 she has been working as a PhD student at Utrecht and Wageningen University. In 2005, she was granted with a short-term stay at the Roslin Institute (UK) from EADGENE to work with Prof. Stephen Bishop on a party of her PhD. Since 1st of January 2006 she is employed at Hybro B.V. as a Geneticist (R&D).