Immunogenetic analysis of natural antibody isotypes in laying hens

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Abstract

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Worldwide, especially in Europe, poultry industry is undergoing important changes including ban of the battery housing system and prohibition of beak trimming. The former can facilitate more spread of infectious diseases, and the latter will contribute to higher mortality because of severe feather pecking. Furthermore, given the growing social concern about food safety and human health, abundant use of antibiotics will either be prohibited or restricted. These changes further emphasize the importance of implementing general disease resistance in layers breeding goals next to maintaining high production. The aim of this thesis was to find proper traits which are associated with laying hens survival, and reveal genetic architecture and background underlying the traits. Natural antibody (NAb), which are the antibodies present in normal healthy animals in the absence of a deliberate antigen exposure are an important humoral part of innate immunity. The relationships between survival and NAb isotype titers were firstly investigated by the logistic regression analysis in a population of laying hens from 12 purebred lines. The results indicated that NAb, especially the IgM isotype titers at young age was predictive for survival of a laying period. Genetic parameters of NAb isotypes IgM and IgG titers were estimated in the same population. The estimation showed that both NAb isotypes are moderate to high heritable traits which were possible to breed for. An association study revealed different QTL or SNP markers for NAb isotypes titers. The majority of the commercial laying hens are crossbred. Therefore, the relationships between NAb isotype titers and survival were further investigated in crossbred laying hens. However, a consistent relationship as in the purebred was not found. This confirmed the speculation that non-health-related causes of mortality (severe feather pecking) overruled the anticipated relationships between NAb isotype titers and survival in birds with intact beaks. Overall, the present studies indicate that it is possible to implement NAb especially the IgM isotype titers into the breeding goals of laying hens to improve the health-related survival.

To my family and all my teachers...

Contents

1

General introduction

1.1 Introduction

1.1.1 Achievement of poultry breeding in the past decades

It is not simple to state since when the human beings started to keep chickens for eggs and meat. However, what we observed is that, during the past decades since late 1930s, together with improvements in nutrition and management, commercial animal breeding companies have successfully and dramatically improved the production performance of this domestic animal, either egg production in layers or meat production in broilers (Havenstein et al., 2003). Specialized breeds and lines are also generated during the intensive and effective artificial selection process for many economically important traits. In laying hens, all white hens are based on the White Leghorn breed that originated from Livorno in Tuscany, Italy, whereas Brown hens are mainly based on Rhode Island Red and White Plymouth rock breeds (Muir et al., 2008). Many commercial laying hen breeds can now produce 300 or more eggs per year, which is three-fold of that at the turn of the 20th century (Ensminger, 1992). The industry continues to improve the efficiency of laying hens production by at least 1% per year. In 2009, an estimated 62.1 million tons of eggs were produced worldwide from a total flock of approximately 6.4 billion laying hens. World egg production in 2013 will likely reach a record of 65.5 million tons (www.wattagnet.com/Outlook_for_egg_production.html).

1.1.2 Challenges for future laying hens breeding

Eggs are an important and relative cheap food resource for good protein and other important nutrients like choline. Along with the continuous increasing worldwide population, ever growing food resource (protein) requirement, as well as the economic profit driving, maintaining the high level of production efficiency is still the major mission for the layers breeding. Genetic improvement in egg production is challenged by the highly canalized nature of the reproduction trait as determined by diurnal photoperiodic constraints (An egg is formed gradually over a period of about 25 hours). Increased early maturity has long been an important characteristic for birds to improve the production by lengthening the laying period. At the present time, most of the commercial laying hens are raised for laying eggs until 80 to 90 weeks of age, depending on the breeds. For the layers breeding companies, an extended laying life of hens is an attractive alternative approach, as it will make better use of layer house facilities, reducing the financial impact and down time involved in replacing pullets. Recently, a new type of production cycle of 100 weeks, during which time the laying hens will be capable of producing 500 eggs

without forced-molting process is expected by Institut de Sélection Animale (ISA), the layer breeding division of Hendrix Genetics. They also showed that there were pure line flocks with these characters when kept in a single-bird cage and in a disease-free environment under the best conditions possible (www.worldpoultry.net/Breeders/General/2011/3/Breeding-for-500-eggs-in-100-weeks-WP008564W/). However, the genetically achievable objective can be compromised under the practical conditions their commercial progeny are expected to perform in. Keeping up the health status and survival ability is required to permit the full exploitation of genetic potential of egg laying persistency. Therefore, the goal for the new life cycle in laying hens also emphasizes the livability of the birds with aging.

Next to the top mission of maintaining the high level of production efficiency, the worldwide poultry industry is currently challenged to improve animal welfare as well as food quality and safety. This is likely to have an impact on future laying hens breeding practice.

First, consumers especially those from European Union (EU) are claimed to have increasing preferences for the welfare of production animals. Animal welfare in commercial poultry production is therefore becoming an important topic and receives more legislative attention. In some non-EU countries, an increasing focus on farm animal welfare is also driven by export opportunities for poultry meat (van Horneand and Achterbosch, 2008). The major concerns of welfare of laying hens in EU were the space allowance per hen and the status on mutilations (e.g. beak trimming). To accommodate societal concerns about animal welfare for the laying hens, the conventional cage (battery) housing system where the hens have limited space to express natural behavior like sand bathing and wing flapping, was banned prior to 2012 in Germany, the Netherlands, Austria, and Sweden (European Union Council Directive 1999/74/EC). As a result, all hens are kept in alternative barn housing systems (large enclosures with litter on the floor and freedom of movement for the birds within the poultry house). This system permits the laying hens to have contact with more mates. It is not feasible to avoid the exposure of chickens to (entero) bacteria such as *E. coli* in the floor system (Cavero et al., 2009). The close contact with mates can facilitate the spread of infectious diseases. Therefore the floor housing system will challenge the birds with an increasing threat from infectious diseases and an increase in mortality (Blokhuis et al., 2007). Furthermore, ban of beak trimming will also contribute to a higher mortality because intact beaks enable severe feather pecking and cannibalism.

Second, the close links between the triad of animal disease, food security and public health is more and more realized. Animal diseases affect production and productivity of animals. Additionally, some animal diseases transmissible to man (zoonosis) also affect public and human health as evidenced by outbreak of emerging diseases such as different strains of avian influenza. Worldwide, animal diseases were mainly prevented by vaccination, feed addition like probiotics or cured by the use of antibiotics drugs. Low dose antibiotics have, however, also been widely used as growth promoters in animal food. The industry does benefit from these measures, but also pay a high price as (1) the application of drugs are expensive and labor is needed, (2) the emergence of virulent and drug resistant pathogens call for continuous development of new drugs or vaccines, and (3) use of antibiotic feed additives in animals feeds may contribute to the problem of antibiotic resistance in human medicine (Castanon, 2007). Abundant use of antibiotics will either be prohibited or restricted.

Mortality due to diseases is responsible for 10 to 20% of the gross production value in the poultry industry and likely higher in the developing countries (FAO, 2011). To some extent, the challenges mentioned above in the industry weaken the external protection (e.g. drug treatment) of the animals from diseases or other disturbance, while internal genetic merit about maintaining health is therefore highly required. In other words, laying hens are appreciated which are resistant to as many as possible diseases, maintain higher production levels, need less veterinarian and drug treatment and live a longer productive life are appreciated. From the farmers' point of view, less disease and more healthy animals means less production cost and more economic profit; from the consumers' point of view, these means more safe food from the production chain. More attention is urged to be paid by poultry breeding companies to enhancing general health or disease resistance and welfare of the laying hens, next to the production traits.

1.1.3 Improve animal health and survival by breeding for a better immunity

The basic definition of health is the absence of diseases (Gunnarsson, 2006) and other physiological disorders. Survival is highly related with the ability of the living organism to cope with the diseases or other negative environmental disturbances. Therefore it is the direct reflection or outcome of animal health status. However, survival data can only be noted when the animal died. A functional immune system is the individual's defense mechanism to fight against diseases and thus of vital importance for the animal's health status and survival. Besides, the involvement of

immune components has been established in a variety of metabolic and behavioral disorders (Biscarini et al., 2010a; Brunberg et al., 2011; Buitenhuis et al., 2004; Hughesand and Buitenhuis, 2010; Parmentier et al., 2009). Compared with environmental factors like management and nutrition, breeding for a better immunity has permanent and cumulative genetic improvement for health. Finding immunity parameters which are also predictive for survival to breed for is therefore meaningful. Better still, once the genetic variations associated with these traits are identified and validated, marker-assisted selection (MAS) (Landeand and Thompson, 1990) or genomics selection (Meuwissen et al., 2001) can accelerate the improvement of animal health. The identification of genetic variations in the causative genes, on the other hand, will facilitate a better understanding the biological mechanism underlying the traits.

The immune system (Box 1.1) is complex and composed of many ingredients. Hence, finding immunity-related traits which are predictive for survival also represent some difficulties. Normally, an innate and an adaptive part of immune system, which perform different roles, are distinguished. Adaptive immunity has proven to be of considerable practical importance, as witnessed by the extensive use of vaccines in animal farming. The adaptive immune response to some specific pathogens or vaccines has been considered in selection for disease resistance and survival (Cavero et al., 2009). The mechanisms as well as the genetic background of adaptive immunity have been widely studied. To date, 420 QTL have been reported to be associated with health or disease related traits in chicken (www.animalgenome.org/cgi-bin/QTLdb/GG/index). Examples include QTL affecting the resistance (Heifetz et al., 2009) and susceptibility (Heifetz et al., 2007) to Marek's disease in laying hens, and QTL for resistance to Salmonella in broilers (Ghebremicael et al., 2008). However, the cost of pathogen-challenge trials for these studies is very high and presents bio-security risks. The birds selected for resistance for one specific pathogen are not always resistance for other pathogens, while the laying hens delivered from the breeding company worldwide have to cope with different hygienic circumstances with different pathogens and disease pressures. Those QTL are related with some specific disease or pathogen. It is not practical to include all, but also difficult to make a decision about which ones should be considered in a genetic selection program (Lamont, 1998). In contrast to adaptive immunity, the innate immunity is ready to act and can stop infections before they cause diseases. However, the potential of improvement of innate immunity for health and survival was not investigated in great detail.

1.1.4 Natural antibody and isotypes

Natural antibody (NAb), which are the antibodies present in the circulatory system of normal healthy animals in the absence of a deliberate antigen exposure like vaccination or infection, are claimed to be an important humoral part of innate immunity (Avrameas, 1991). NAb constitute a large fraction of the serum immunoglobulins. Every species tested so far including humans (Chou et al., 2009), mice (Ochsenbein et al., 1999), rats (Natori et al., 1981), rabbits (Gerencer et al., 1998), fish (Sinyakov et al., 2002), and poultry (Neu et al., 1984) all produce NAb. It is widely accepted that NAb are produced by B-1 B cells in humans and mice, which develop early in ontogeny (Kohler et al., 2003; Ochsenbein et al., 1999), while specific antibodies (SpAb) are produced by B-2 B cells. The exact origin and development of NAb in chickens is still a mystery. They may share the similar mechanism as humans and mice. However, they may also possess its distinct mechanism of producing NAb, because CD5, which is a glycoprotein used to distinguish B-1 cell and B-2 cells (Kasaian and Casali 1993), has been found on all chickens B cells (Koskinen et al. 1998).

Box 1.1 The immune system

An immune system is present in all species in the animal kingdom and is a defense against intruding organisms, molecules and malignant cells. In a broad sense, the immune system in birds is no different from the immune system found in mammals. The immune system can be divided into two parts: the innate (non-adaptive) and the acquired (adaptive) immune system. The term "innate" refers to the fact that this type of host defense is always present in healthy individuals, ready to engage on blocking the entry of microbes and to rapidly eliminate microbes that do succeed in entering host tissues. The components of innate immune system include phagocytes, natural killer cells, complement system, cytokines, and other plasma proteins. Because of the property of NAb, it is also regarded as a component of innate immunity. The innate response compromises many functions and acts as a first line of defense against infections. In contrast, it takes longer time for the adaptive immune response to be initiated, but it is highly specific for a particular antigen. T and B lymphocytes and specific antibody are the main functional components of adaptive immunity.

Although NAb have often been neglected as the so-called background serum antibodies without significant relevance (Korver et al. 1984), an increasing amount

of evidence indicates that NAb have broad reactivity against foreign antigens and play multiple roles in health and disease (Lutz, 2012).

Box 1.2 Keyhole limpet hemocyanin (KLH)

KLH is a large metalloprotein found in the hemolymph of the giant keyhole limpet (*Megathura crenulata*), which naturally lives off the coast of California from Monterey Bay to Isla Asuncion off Baja California, Mexico (Harris and Markl, 1999). Prior exposure or sensitization to this protein is considered unlikely for chickens. KLH was previously used as an example of a naïve antigen for detecting NAb in laying hens (Parmentier et al., 2004a; Star et al., 2007). These NAb are so-called overt NAb (Cheng and Chamley, 2008). There are also cryptic NAb which are usually directed to self-antigen like egg white protein (Parmentier et al., 2004a).

First, NAb has moderate affinity, and are typically poly-reactive (Avrameas, 1991). NAb may provide a pre-existing antibody reactivity that acts as an early defense and allows animals to rapidly recognize pathogens that animals have not previously encountered. This reactivity prevents and delays the spread of the pathogens to vital organs and improves immunogenicity through enhanced antigen-trapping in secondary lymphoid organs (Ochsenbein et al., 1999). Second, NAb are indicated to perform crucial homeostatic housekeeping functions in the maintenance of physiological and immunological homeostasis (Anania et al., 2010), protecting the body against stress-induced altered self-antigen immunity (Cheng, 1998; Ehrenstein and Notley, 2010; Lutz, 2007; Lutz et al., 2009). Immune surveillance of natural IgM by reorganization and elimination of precancerous and cancerous lesions was also highlighted (Vollmers and Brandlein, 2006). Third, the innate immunity is also the stimulus for the adaptive immunity (Iwasaki and Medzhitov, 2004). An effective immune system often requires the coordinated action of both innate immunity and adaptive immunity. NAb also cooperate with and are additional to the specific immune system (Baumgarth et al., 2005) by capturing and presenting antigens to T helper cells. Various specific immune responses in mammals, humoral- (Kohler et al., 2003; Thornton et al., 1994; Tomer and Shoenfeld, 1988) as well as cellular immune responses (Stager et al., 2003) are enhanced by or positively correlated with titers of NAb. Similar observations are described in poultry (Lammers et al., 2004). In dairy cattle, the reduced presence of clinical mastitis is also associated with higher level of NAb detected in milk (Ploegaert, 2010). NAb are such a non-redundant part of the immune system that it is anticipated that NAb titers may reflect the ability of an animal to keep healthy

and survive better. In the absence of NAb, there is delayed adaptive immune response and increased mortality in mice caused by influenza (Baumgarth et al., 2005). Star et al., (2007) showed that titers of total NAb binding keyhole limpet hemocyanin (KLH) (Box 1.2) were indicative for a higher probability that chickens survive a laying period.

Alike SpAb, NAb can be either of the IgM, IgG (IgY), and IgA isotypes (Box 1.3) in birds. IgM is the principal isotype although IgG and IgA were also reported. Evolution of the various Ig classes has made a significant contribution to functional diversity in terms of antigen processing and recruitment of effector mechanisms (Janeway et al., 2001), such as a more protective preventive barrier function (IgM), or a responsive status (IgG) after infection or sensitization. In addition, IgM may modulate the production of IgG antibodies (Ehrenstein and Notley, 2010). In the study of Star et al. (2007), only total NAb titers were studied without distinguishing different isotypes. These various functions suggest that NAb isotypes may be differently related to health and survival.

Box 1.3 Antibody isotype

Antibodies are typically made of basic structural units-two large heavy chains and two small light chains. Antibodies in placental mammals are grouped into five different isotypes known as IgA, IgD, IgE, IgG, and IgM, based on which of the five kinds of heavy chain they possess (α, δ, ε, γ, and μ, respectively). Antibody isotypes differ in their biological properties, functional locations and ability to deal with different antigens (Crawley and Wilkie, 2003, Woof and Burton, 2004). In avian, three antibody isotype were recognized namely IgY, IgM (majority isotypes in blood), and IgA (majority isotype in lung and gut). IgY is the avian counterpart to mammalian IgG, although differs both structurally and functionally. B1 cells are a significant source of natural serum IgM. Isotype switching changes B cell's production of antibody from one isotype to another (from IgM to IgG or IgA) by changing the constant region of heavy chain (Market and Papavasiliou, 2003).

To be used as selection criteria for health and survival, except for the immune merits, NAb isotypes should also be checked for the following characters: (a) large variation in the population, which is the basis of genetic gain; (b) are moderate to high heritable, so that individual selection can be applied and relevant genetic gain due to selection can be expected; (c) a target value or clear trend is clear. Experiment data are required to show if high, low or a median level of the NAb

isotype is favorable; (d) easy and accurate to measure. Although genomic selection is revolutionizing animal breeding by estimating the breeding value based on associated SNP markers instead of based on phenotype records and pedigree information (Meuwissen et al., 2001), phenotyping in the reference population is still necessary. For future routine screening of animals it is important that the parameters can be determined easily, and in samples that can be obtained without causing severe consequence; and (e) correlation with other economic traits is clear, this point will be further elucidated in the following paragraph.

1.1.4 Balanced breeding for laying hens

As the potential trait to be implemented into the breeding goal to improve general health and survival of the birds, NAb are also expected or reported to be related with other economics traits. First of all, the final commercial products of laying hen industry are eggs, which is also the reproduction trait of laying hens. In the breeding goal of layer dam lines the main focus is on female reproduction traits. In the breeding goal of layer sire lines relatively large weight is placed on egg quality and male reproduction traits. As two aspects of fitness of animals, reproduction and maintain health and survival sometimes show a negative genetic trend (Goddard, 2009). Second, severe feather pecking behavior is a typical welfare problem and causes much economic loss in laying hens. NAb titers were reported to be related with feather pecking (Biscarini et al., 2010a). Third, common genetic background for NAb isotypes titer and other adaptive immune traits was also revealed (Biscarini et al., 2010b). Higher levels of NAb are found in birds selected for high SpAb responses to sheep red blood cell (SRBC) (Parmentier et al., 2004b). However, the trade-off between NAb and SpAb production was clearly shown in reptiles (Sandmeier et al., 2012). In laying hens, long-lasting response to vaccination procedure are more valuable (enhanced adaptive immunity), because the laying hens are reared for a relative long life. Given their importance to profitability, these traits of concern in laying hens should not be compromised when trying to complement general health and survival in the breeding goals. Therefore, it is also necessary to study the association between the indicator traits to continually improve these traits, in a balanced way, for general health and survival and these traits of concern in laying hens.

1.2 Aim and outline of this thesis

The research described in this thesis aimed to (1) find out proper immune parameters which are associated with and predictive for survival of laying hens,

and which can be implemented in the breeding program for improved survival of laying hens (**chapters 2** and **chapter 4**), (2) estimate the genetic parameters and reveal the associated genetic regions of the predictive parameters (**chapter 3**), (3) investigate the relationship between the parameters and feather pecking behavior (**chapter 5**).

In **chapter 2** of this thesis, serum titers of NAb isotypes IgM and IgG binding KLH in a population of laying hens from 12 purebred lines of two commercial breeds, Rhode Island Red and White Leghorn was assessed. These trait variations were estimated between lines as well as within an individual genetic line. Multivariable multilevel logistic regression models were used to investigate the relationships between survival and titers of NAb isotypes of laying hens during one whole laying period. As covariates, genetic origin (line) and body weight of the laying hens were also included in the analyses. The opportunity for selection on a trait depends on the heritability, which measures the amount of additive genetic variation in a trait. Therefore in **chapter 3**, we firstly estimated the genetic parameters of NAb isotypes IgM and IgG titers binding KLH. Then an association study was performed to identify different QTL or SNP markers for NAb isotypes titers. A population of laying hens from nine commercial purebred lines was used in the analysis, adopting an across-line approach with testing of SNP-by-line interaction. The results will help to better understand the genetic control of levels of innate immunity, thus disclosing opportunities to breed for higher survival in laying hens. The majority of commercial laying hens are crossbred. In **chapter 4**, genetic parameters of NAb isotypes were estimated and relationships between survival and NAb isotypes levels in beak trimmed and non-beak trimmed crossbred laying hens were investigated. The genetic link between behavioral disorders and innate immunity was reported. However, negative indirect select effects, for example a more severe feather pecking behavior is not wanted by breeders along with the selection for high NAb level. Therefore, in **chapter 5**, genetic architecture of laying hens welfare related traits, feather pecking was analyzed using a traditional linear model and a model combining direct and associative effect. Furthermore, the relationships between performing feather pecking behavior and NAb isotype titers were also investigated. Finally, in the general discussion described in **chapter 6**, the main findings of the present thesis are discussed and comments on the breeding strategy to meet future laying hens breeding practice for improved animal welfare and production traits are proposed.

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2

Natural antibody isotypes titers as predictors of survival in purebred laying hens

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Abstract

To identify possible relationships between survival and titers of natural antibody (NAb) isotypes in serum of laying hens, birds from 12 purebred layer lines of two commercial breeds, Rhode Island Red (R breed, *n* = 524) and White Leghorn (W breed, *n* = 538), were monitored for survival during one laying period (from 20 until 70 weeks of age). Titers of NAb isotypes IgM and IgG binding keyhole limpet hemocyanin (KLH) in serum were measured at 20, 40, and 65 weeks of age, respectively. Overall, the titers of IgM and IgG binding KLH decreased with aging. At the same age, lines within breed showed significantly different titers of isotypes ($P < 0.001$). Multivariable logistic regression analysis showed that NAb isotypes titers at 20 weeks of age were associated to survival of 20 to 40 weeks of age. In the R breed, odds ratios of 0.56 ($P < 0.001$) for IgM and 0.72 ($P = 0.02$) for IgG were estimated; in the W breed, these were 0.74 ($P < 0.01$) and 0.99 ($P = 0.95$) for IgM and IgG respectively. We conclude that titers of NAb especially the IgM isotype binding KLH at 20 weeks of age are indicative for survival during the laying period. The higher the titers of NAb isotypes, the higher the probability of layers to survive.

Key words: survival, natural antibody, isotype, laying hen

2.1 Introduction

A functional immune system is the individual's defense mechanism to fight diseases and thus of vital importance for the animal's survival. Antibodies are part of the humoral immune system. The health status of an animal when encountering an infectious agent is closely associated with its ability to produce specific antibodies (SpAb) to the infectious agent or to previous vaccination (Yonash et al., 2001). The drawback of SpAbs is that protection is limited to specific pathogens and specificity also depends on previous immunization or exposure. Every species tested so far including humans (Chou et al., 2009), mice (Ochsenbein et al., 1999), rats (Natori et al., 1981), rabbits (Gerencer et al., 1998), fish (Sinyakov et al., 2002) and poultry (Neu et al., 1984) produces natural antibodies (NAb). In contrast to SpAbs, NAb have been defined as immunoglobulins that are secreted by B-1 cells (in mammals) and are present in animal's circulation system in the absence of any corresponding or earlier antigen exposure (Avrameas, 1991). NAb were shown to be an essential part of the first line of defense (Ochsenbein and Zinkernagel, 2000). NAb have low affinities but broad specificities to both foreign and self-structures.

As the humoral arm of innate immunity, NAb may have been conserved by natural selection. They must be of benefit to the host in a general context (Chou et al., 2008). First, NAb may provide a pre-existing antibody reactivity that acts as an early defense and allows animals to rapidly recognize pathogens that animals have not been previously encountered. This reactivity prevents and delays spread of the pathogen to vital organs and improves immunogenicity through enhanced antigen-trapping in secondary lymphoid organs (Ochsenbein et al., 1999). Second, NAb are indicated to perform crucial homeostatic housekeeping functions in the maintenance of physiological and immunological homeostasis, protecting the body against stress-induced altered self-antigen immunity (Cheng, 1998; Lutz, 2007; Lutz et al., 2009; Ehrenstein and Notley, 2010). An effective immune system often requires the coordinated action of both innate immunity and adaptive immunity. Natural antibodies also cooperate with and are additional to the specific immune system (Baumgarth et al., 2005). Various specific immune responses in mammals, humoral- (Tomer and Shoenfeld, 1988; Thornton et al., 1994; Ochsenbein et al., 1999; Kohler et al., 2003) as well as cellular immune responses (Stäger et al., 2003) are enhanced by or positively correlated with titers of NAb. Similar observations are described in poultry (Lammers et al., 2004).

NAb are such a non-redundant part of the immune system that it is anticipated that NAb titers may reflect the ability of an animal to keep healthy and survive better. A

previous study showed that total titers of NAb binding keyhole limpet hemocyanin (KLH) were indicative for a higher probability that chickens survive a laying period (Star et al., 2007).

Antibodies consist of different varieties or classes known as isotypes: IgG (IgY in aves), IgM, and IgA. In mammals, most NAb are IgM (Boes et al., 1998b; Boes, 2000), but examples of IgG and IgA isotypes of NAb have also been reported (Avrameas, 1991; Boes, 2000; Simell et al., 2008). NAb isotypes may differ in immune functions, such as a more protective preventive barrier function (IgM), or a responsive status (IgG) after infection or sensitization. In addition, IgM may modulate the production of IgG antibodies (Ehrenstein and Notley, 2010). These various functions suggest that NAb isotypes may be differently related to survival.

The aim of this study was to assess serum titers of NAb isotypes IgM and IgG binding KLH and use the multivariable multilevel logistic regression models to evaluate the relationships between survival and titers of NAb isotypes of laying hens during one whole laying period. As covariates, genetic origin (line) and body weight of the layers were also included in the analyses.

2.2 Materials and methods

2.2.1 Chickens, housing, and feed

A total number of 1,062 layers were used for this study. Within this population, 12 purebred layer lines (Hendrix Genetics, Boxmeer, The Netherlands) from 2 breeds could be distinguished: 6 Rhode Island Red (R breed) lines (B1, B2, B3, BA, BB and BE) and 6 White Leghorn (W breed) lines (W1, WA, WB, WC, WD and WF), respectively. The housing, feed and immune procedures were described in an earlier publication (Star et al., 2007). Average egg production from 25 to 69 weeks of age during the laying period was estimated for the 12 layer lines, ranging from 243 (line BE) to 255 (line B3) in the R breed and 251 (line WD) to 263 (line WF) in the W breed (Star, 2008). The numbers of samples used for analyses at different ages are shown in Table 2.1.

2.2.2 Study Design

The observational period was from 20 until 70 weeks of age of the layers. For the chickens that died during this laying period, the day of survival was registered, but cause of death was not determined. Body weights of the chickens were determined at 22 and 40 weeks of age. Blood samples were taken by wing vein puncture at 20,

40, and 65 weeks of age for the measurement of IgM and IgG titers binding KLH which the chickens had not encountered before nor were likely to encounter during life.

Table 2.1 Number of birds at the start of the laying period (17 weeks) and number of samples used at each sampling periods (20, 40, and 65 weeks, respectively); average BW (± SD) of the birds at 22 and 40 weeks; average survival days (± SD) and survival over the whole laying period of 12 layer lines.

	Birds (n)				Body weight (g)		Survival days (d)	Survival[2] (%)
Line[1]	17 weeks	20 weeks	40 weeks	65 weeks	22 weeks	40 weeks		
B1	91	80	82	70	1,525±122	1,830±179	326±78	76.9
B2	86	72	76	66	1,461±106	1,966±220	333±84	84.9
B3	84	57	71	79	1,560±156	2,066±183	354±49	92.9
BA	89	88	80	68	1,541±161	2,166±205	330±83	80.9
BB	91	91	80	64	1,536±174	1,991±240	329±76	75.8
BE	97	69	84	73	1,610±155	2,029±165	325±81	74.2
W1	85	83	76	64	1,219±98	1,714±152	326±81	75.3
WA	82	79	70	60	1,282±143	1,622±151	345±67	89.0
WB	90	37	57	53	1,345±124	1,662±192	327±83	75.6
WC	97	74	82	61	1,290±152	1,655±149	307±99	64.9
WD	88	88	85	69	1,215±118	1,625±157	336±70	83.0
WF	82	54	53	52	1,244±138	1,776±165	337±80	87.8

[1]Lines B1, B2, B3, BA, BB, and BE are from the Rhode Island Red breed and lines W1, WA, WB, WC, WD, and WF are from the White Leghorn breed.
[2]Number of birds survived until the end of the whole laying period (69 weeks) / number of birds at the start of the laying period (17 weeks).

2.2.3 NAb isotypes IgM and IgG

Titers of NAb isotypes IgM and IgG binding KLH were determined in individual serum samples by an indirect enzyme-linked immunosorbent assay (ELISA) as follows. Flat-bottomed 96-well medium binding ELISA plates were coated with 100 µL coating buffer (pH 9.6) containing KLH (2 µg/mL, MP Biomedicals Inc., Aurora, OH), and incubated at 4°C overnight. Duplicate standard positive serum samples were stepwise diluted in columns 11 and 12 per plate, respectively. After subsequent washing the plates were filled with 100 µL of PBS containing Tween 20 (0.05%) and horse serum (0.5%) per well. Serum samples were stepwise fourfold diluted (1 : 30, 1 : 90, 1 : 270, and 1 : 810), and the plates were incubated 1 hour at room temperature (25 °C). After washing, plates were incubated with 1 : 20,000 diluted rabbit-anti-chicken IgM labeled with peroxidase (RACh/IgM/PO), or 1 : 40,000 diluted rabbit-anti-chicken IgG-Fc (RACh/IgG/Fc/PO), respectively (Bethyl Laboratories, Texas, U.S.A.), and incubated 1.5 hour at room temperature (25 °C).

After washing, binding of the antibody isotypes in the serum sample to KLH was visualized by adding 100 µL substrate (70 µg/mL tetramethylbenzidine and 0.05% H2O2). After 10 minutes, the reaction was stopped with 50 µL 2.5 N H2SO4 solution. Extinctions were measured with a Multiskan (Labsystems, Helsinki, Finland) at wavelength of 450 nm. Titers were calculated based on log3 values of the dilutions that gave extinction closest to 50% of EMAX, where EMAX represents the mean of the highest extinction of the standard positive serum (column 11 and 12) present on each plate (in general this will be at the lowest dilution).

2.2.4 Statistical analysis

Statistical analyses were performed using SAS 9.1.2 (SAS Institute, 2004). Effects were considered significant at $P < 0.05$.

A one-way analysis of variance (ANOVA) was performed to study differences in titers of IgM and IgG binding KLH and body weights between breeds and between lines within breed. When ANOVA identified an overall difference between lines, a multiple comparison test (Bonferroni Test) was conducted to examine which lines differed significantly from each other within breed.

Correlations between each isotype titers at 20 and 40 weeks, between 40 and 65 weeks, and between 20 and 65 weeks of age were estimated for every line by Pearson product-moment correlation.

The laying period was divided into 3 parts (20 to 40 weeks of age, 40 to 65 weeks of age, and 65 to 70 weeks of age) based on the time of blood sampling. Within breed, multivariable multilevel logistic regression analyses were used to assess the relationship between 1) survival (binary variable taking the values 0 for survived and 1 for non-survived) from 20 to 40 weeks of age and titers of IgM or IgG antibodies binding KLH at 20 weeks of age; 2) survival from 20 to 65 weeks of age and titers of IgM or IgG antibodies binding KLH at 20 weeks of age; 3) survival from 40 to 65 weeks of age and titers of IgM or IgG antibodies binding KLH at 40 weeks of age. The relationship between survival after 65 weeks of age and IgM or IgG antibodies binding KLH at 65 weeks of age was not analyzed because of the low mortality (0.79%, 6 birds) in the last 5 NAb of the laying period. As covariates, line and bodyweight were included in the analyses.

The continuous variables IgM, IgG titers and body weights were inspected for linearity in the log-odds by dividing them into classes. The Likelihood Ratio Test was used for the significance of variables. Non-significant effects ($P > 0.05$) were removed from the model one by one starting with the effect showing the highest *P*-

value. If a removed effect was deemed a confounder (i.e. one or more regression coefficients of the remaining variables relatively changed over 25%) it was forced back into the model. We also tested the interactions terms between isotype titers and body weight or lines. A population averaged model (generalized estimating equations, GEE) was used to adjust for a potential cage effect on survival by specifying cage as a random effect and using an exchangeable correlation structure (Hanley et al., 2003). The fit of the logistic models was assessed by the Hosmer and Lemeshow Goodness-of-Fit Test in a model without the cage effect (Hosmer and Lemeshow, 1989). Outcomes of logistic regression analyses were presented as odds ratios, which indicate the ratio of risks to die dependent on the titers of IgM and IgG binding KLH, body weight and line.

2.3 Results

2.3.1 NAb isotypes IgM and IgG binding KLH

Average titers of IgM and IgG binding KLH in laying hens at 20, 40, and 65 weeks of age differed between R breed and W breed (Figure 2.1). For IgM, significant differences ($P < 0.001$) between both breeds existed at 20, 40, and 65 weeks of age (Figure 2.1A); for IgG, the difference was significant ($P < 0.001$) at 20 weeks of age (Figure 2.1B). The average titers of IgM and IgG antibodies in serum binding KLH decreased with age in both breeds.

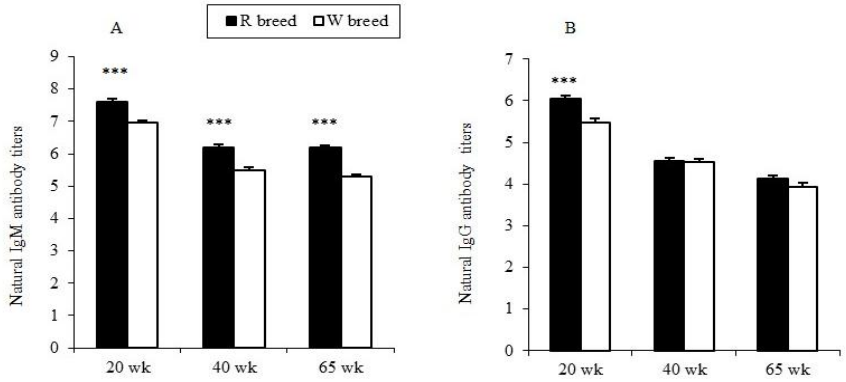

Figure 2.1 Average titers of IgM and IgG antibodies in serum binding KLH in two breeds: White Leghorn (W breed) and Rhode Island Red (R breed). ***$P < 0.001$.

Figure 2.2 shows the average titers of IgM and IgG antibodies binding KLH in laying hens at 20, 40, and 65 weeks of age for each line. From the six R lines, the B1 and BB lines presented the highest and lowest titers of IgM, respectively, and the B1

and BE lines showed the highest and lowest titers of IgG at each age. From six W lines, the WF and WB lines presented the highest and lowest titers of IgM, respectively; and lines WC and WB respectively, presented the highest and lowest titers of IgG at each age. The variation of average IgM titers between lines within the W breed was larger than within the R breed. In 9 of 12 lines, except for WD, BE and B3 from 40 to 65 weeks of age, IgM titers decreased with age. In 9 of 12 lines, except for lines WA and WF from 20 to 40 weeks of age, and for line W1 from 40 to 65 weeks of age, IgG titers binding KLH decreased with age.

Within almost all lines, significant but weak correlations were found for titers of IgM or IgG antibodies binding KLH between 20 and 40, 20 and 65, 40 and 65 weeks of age, except for the correlation of IgM or IgG between 20 and 40, and 20 and 65 weeks for both IgG and IgM were stronger than the correlations between 20 and 40 weeks or between 20 and 65 weeks (Table 2.2 and Table 2.3)

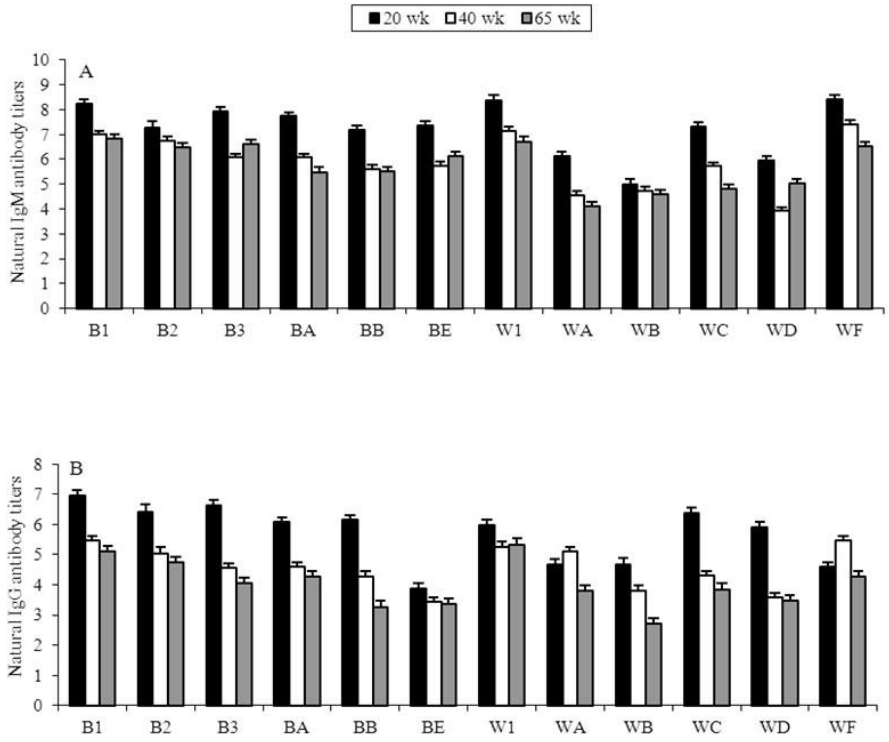

Figure 2.2 Average titers of IgM (Figure 2.2A) and IgG (Figure 2.2B) in serum binding KLH in 12 lines: six White Leghorn lines: B1, B2, B3, BA, BB and BE and six Rhode Island Red lines: W1, WA, WB, WC, WD and WF.

2.3.2 Body weight

Average body weight of the 12 purebred layer lines at 22 weeks of age varied between 1,215 g (line WD) and 1,610 g (line BE). Average body weight of the 12 purebred layer lines at 40 weeks of age was between 1,622 g (line WA) and 2,166 g (line BA) (Table 2.1). R breed birds showed significantly higher body weight than W breed birds at both ages ($P < 0.001$).

Table 2.2 Pearson correlation coefficients (r) and *P*-values of IgM in serum- binding keyhole limpet hemocyanin (KLH) in 12 purebred lines between the 3 sampling periods (20, 40, and 65 weeks of age).

	20 and 40 weeks		20 and 65 weeks		40 and 65 weeks	
Line[1]	r	*P*-value	r	*P*-value	r	*P*-value
B1	0.49	< 0.001	0.49	< 0.001	0.59	< 0.001
B2	0.49	< 0.001	0.36	< 0.01	0.62	< 0.001
B3	0.38	< 0.01	0.31	0.02	0.66	< 0.001
BA	0.31	< 0.01	0.21	0.09	0.42	< 0.01
BB	0.56	< 0.001	0.36	< 0.01	0.41	< 0.01
BE	0.46	< 0.01	0.38	< 0.01	0.59	< 0.001
W1	0.42	< 0.01	0.39	< 0.01	0.52	< 0.001
WA	0.33	< 0.01	0.35	< 0.01	0.77	< 0.001
WB	0.41	0.04	0.58	< 0.01	0.43	< 0.01
WC	0.14	0.30	0.06	0.70	0.68	< 0.001
WD	0.56	< 0.001	0.47	< 0.001	0.58	< 0.001
WF	0.10	0.52	0.18	0.25	0.51	< 0.01

[1]Lines B1, B2, B3, BA, BB, and BE are from the Rhode Island Red breed and lines W1, WA, WB, WC, WD, and WF are from the White Leghorn breed.

Table 2.3 Pearson correlation coefficients (r) and *P*-values of IgG in serum-binding keyhole limpet hemocyanin (KLH) in 12 purebred lines between the 3 sampling periods (20, 40, and 65 weeks of age).

	20 and 40 weeks		20 and 65 weeks		40 and 65 weeks	
Line[1]	r	*P*-value	r	*P*-value	r	*P*-value
B1	0.43	< 0.001	0.49	<0.001	0.63	< 0.001
B2	0.40	< 0.01	0.40	< 0.01	0.45	< 0.01
B3	0.22	0.12	0.06	0.72	0.71	< 0.001
BA	0.43	< 0.001	0.37	< 0.01	0.56	< 0.001
BB	0.47	< 0.001	0.35	< 0.01	0.52	< 0.001
BE	0.39	< 0.01	-0.04	0.79	0.59	< 0.001
W1	0.12	0.30	0.06	0.61	0.73	< 0.001
WA	0.34	< 0.01	0.52	< 0.001	0.73	< 0.001
WB	0.35	0.08	0.02	0.93	0.56	< 0.001
WC	0.45	< 0.01	0.43	< 0.01	0.67	< 0.001
WD	0.37	< 0.01	0.19	0.11	0.70	< 0.001
WF	0.30	< 0.01	0.26	0.07	0.73	< 0.001

[1]Lines B1, B2, B3, BA, BB, and BE are from the Rhode Island Red breed and lines W1, WA, WB, WC, WD, and WF are from the White Leghorn breed.

2.3.3 NAb IgM and IgG titers predictive for probability to survive

Survival of the 12 purebred layer lines in this study varied between 64.9% (line WC) and 92.9% (line B3) (Table 2.1) with an overall survival of 79.8%.

The box-whisker plots of distributions of IgM and IgG binding KLH at 20 weeks of age for laying hens of different longevity in the R and W breed are shown in Figure 2.3. The layers of both breeds which lived a longer life showed higher isotype titers, especially IgM binding KLH at 20 weeks of age. The R breed layers which died between 40 and 65 weeks, and the layers which survived the laying period had nearly identical median values of IgM or IgG titers. However, the layers that died between 20 and 40 weeks of age had lower median values of IgM titers (Figure 2.3A).

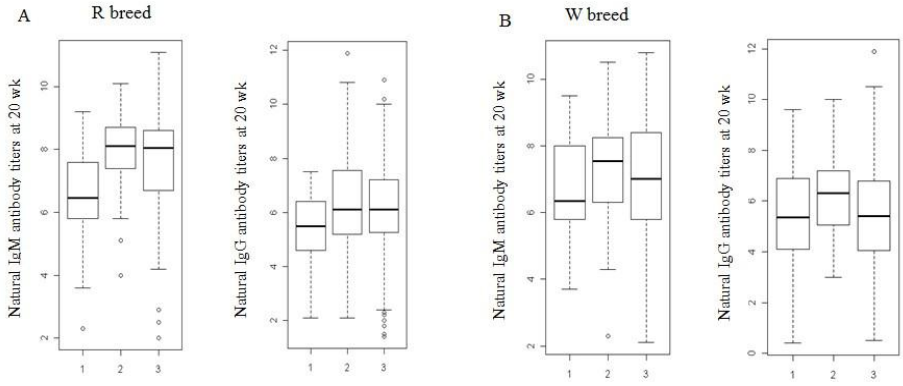

Figure 2.3 Box-and-whisker plots showing the distribution of titers of IgM and IgG in serum binding KLH at 20 weeks of age in 1: layers that died between 20 and 40 weeks, 2: layers that died between 40 and 65 weeks, and 3: layers survived the laying period of R breed (Figure 2.3A) and W breed (Figure 2.3B). The horizontal line in the middle of each box indicates the median, whereas the top and bottom borders of the box mark the 75th and 25th percentiles. The vertical lines above and below the box extend to the 1.5 interquartile range (IQR) of the 75th and 25th percentiles. The single points are potential outliers.

We categorized the layers of both breeds according to isotype titers and compared the survivals of 20 to 40 weeks of age (Figure 2.4). In the R breed, the survival of layers with low titers of IgM binding KLH (< 5.0) was 70.0%, and the survival of layers with high titers of IgM binding KLH (9.0-10.9) was as high as 98.3% (Figure 2.4A). The survival of layers with titers of IgG binding KLH lower than 4.0 was 87.3%, and no animal from the layers with IgG titers binding KLH higher than 8.0 died (Figure 2.4B). In the W breed, the survival of layers with low IgM titers binding KLH (< 5.0) was 91.1%, and the survival of layers with high IgM titers binding KLH (9.0-10.9) was 97.3% (Figure 2.4C). The survival of layers with different range of IgG

titers binding KLH were quite similar (Figure 2.4D). A similar comparison was also carried out for the survivals of 20 to 65 weeks of age (Figure 2.5).

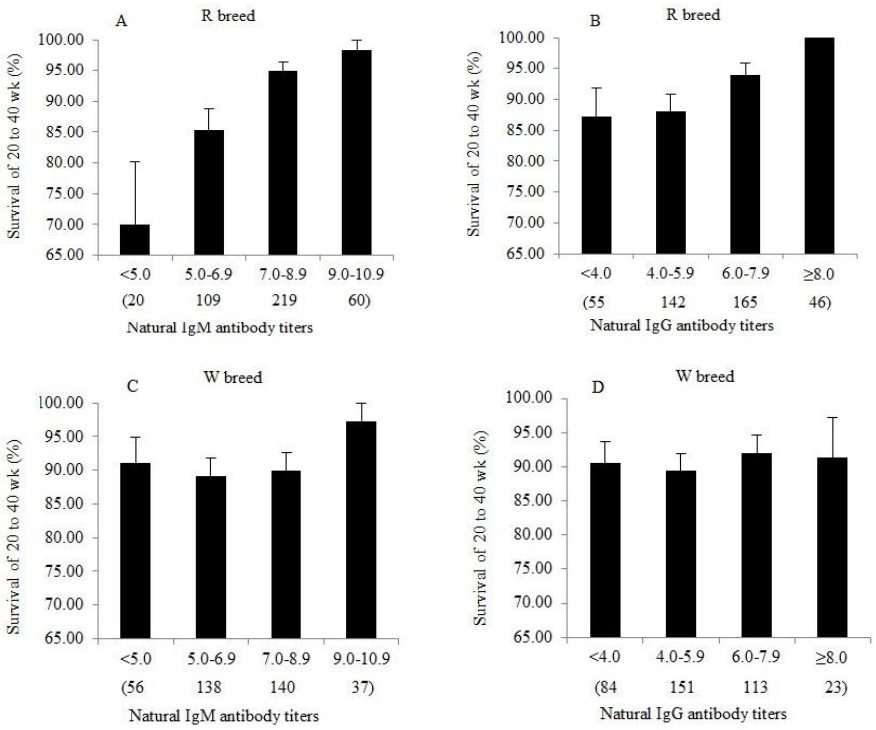

Figure 2.4 Survival (+ SD) of chickens during the first laying period classified by titers of IgM and IgG in serum binding KLH. The numbers between the brackets indicate the number of chickens in each category.

Logistic regression using a population averaged model revealed that the probability to survive the first period (20 to 40 weeks of age) of lay increased with increasing titers of both isotypes at 20 weeks of age. In the R breed, for IgM binding KLH, an odds ratio of 0.56 ($P < 0.001$) was estimated, which means that if titers of IgM in serum binding KLH at 20 weeks of age increase with one unit, the relative change in risk to die during the first period of laying decreases 44%. The odds ratio for IgG binding KLH was 0.72 ($P = 0.02$, Table 2.4), indicating a decrease of 28% in the risk to die per unit increase in IgG. Line had no direct effect on mortality ($P = 0.17$), but when removed from the model, the relative change of the coefficient for IgG titers at 20 weeks of age was large (36%). Body weight at 22 weeks of age was neither a significant factor ($P = 0.09$) nor an important confounder and was removed from the model. The interactions between IgM or IgG and lines were not statistically

significant (*P* = 0.20 and 0.08, respectively), and thus not included in the final model. Population averaged model showed that a minority (11.1%) of all unexplained variation in the survival was due to cage effect. The fit of the ordinary logistic model was sufficient (Chi-square = 10.1, *P* = 0.26).

In the W breed, for IgM binding KLH, an odds ratio of 0.74 (*P* < 0.01) was estimated and for IgG binding KLH, 0.99 (*P* = 0.95, Table 2.4). The odds ratio of body weight (unit is gram) was estimated to be 1.00 (*P* = 0.03,). Line was also determined as a significant factor (*P* = 0.02). The odds ratio was calculated for each line with line W1 as reference (Table 2.4). The interactions between IgM, IgG or body weight and lines were not statistically significant (*P* = 0.41, 0.38, and 0.06, respectively). Population averaged model showed that a minority (14.9%) of all unexplained variation in the survival was due to cage. The Hosmer and Lemeshow Goodness-of-Fit test was not significant (Chi-square = 4.8, *P* = 0.78), which indicated that the model fitted well.

Table 2.4 Multivariable multilevel logistic analysis of IgM and IgG titers in serum binding KLH at 20 weeks of age, line and body weight at 22 weeks of age (BW22) for survival of 20 to 40 weeks of age in two breeds

Breed	Variable[1]	Class	n	Odds Ratio	95% Confidence Interval	*P*-value
R	IgM titers		408	0.56	0.43-0.72	< 0.001
	IgG titers		408	0.72	0.54-0.95	0.02
	Line	B1	68	1.00	Ref	Ref
		B2	58	0.65	0.15-2.77	0.56
		B3	54	0.41	0.071-2.40	0.32
		BA	71	0.59	0.14-2.46	0.47
		BB	91	0.16	0.03-1.03	0.05
		BE	66	0.24	0.03-1.74	0.16
W	IgM titers		371	0.74	0.60-0.92	0.01
	IgG titers		371	0.99	0.79-1.24	0.95
	Line	W1	66	1.00	Ref	Ref
		WA	79	0.12	0.02-0.55	0.01
		WB	37	0.20	0.03-1.21	0.08
		WC	67	0.99	0.27-3.61	0.99
		WD	82	0.21	0.04-0.97	0.05
		WF	40	0.94	0.19-4.70	0.94
	BW22(g)		371	1.00	1.00-1.01	0.03

[1]R breed = Rhode Island Red; 11.1% of all unexplained variation in the R breed is due to cage; W breed = White Leghorn; 14.9% of all unexplained variation in the W breed is due to cage.

The probability to survive the laying period (20 to 65 weeks of age) increased with increasing titers of both isotypes at 20 weeks of age. In the R breed, the odds ratio for IgM was 0.78 (*P* < 0.01). The titers of IgG at 20 weeks of age were not linearly

related to the survival of laying period (20 to 65 weeks) and were therefore categorized in 3 classes, With IgG titers < 4.0 as reference class, the odds ratios for higher IgG titers group were both larger than 1.00 (Table 2.5). This indicated that the chance to survive this laying period decreased when layers had higher titers of IgG. However the association was not statistically significant ($P > 0.05$). Line had no direct effect on mortality ($P = 0.11$), but was forced in the model as it was a confounder for the estimates of IgG. Body weight at 22 weeks of age was neither a significant factor ($P = 0.10$) nor an important confounder. The interactions between IgM or IgG and lines were not statistically significant ($P = 0.75$ and 0.79, respectively). Population averaged model showed that 25.1% of all unexplained variation in the survival was due to cage (Table 2.5). The fit of the ordinary logistic model was sufficient (Chi-square = 3.9, $P = 0.86$).

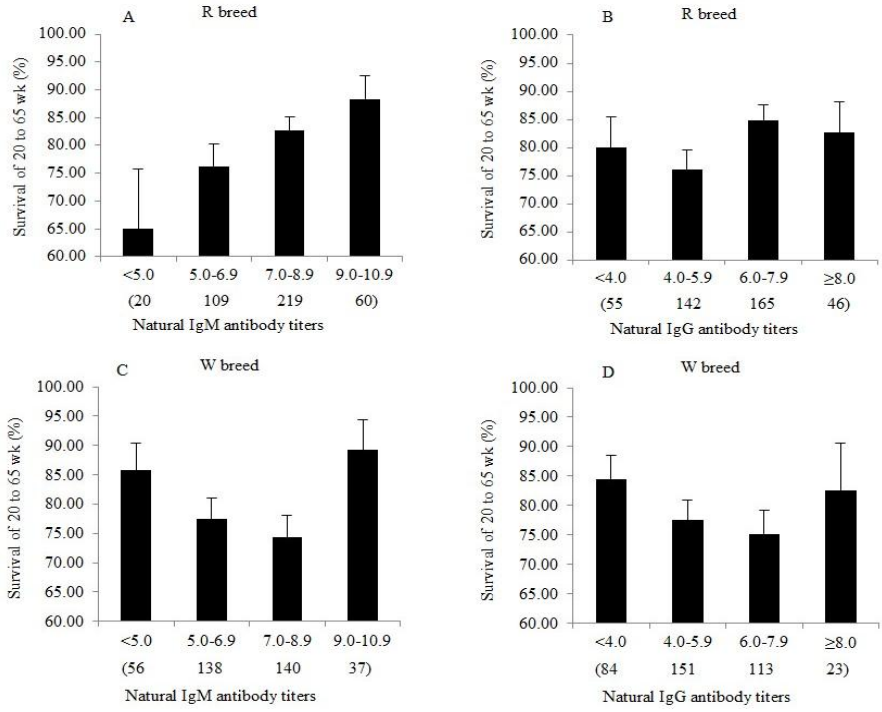

Figure 2.5 Survival (+ SD) of chickens during the whole laying period classified by titers of IgM and IgG in serum-binding KLH. The numbers between the brackets indicate the number of chickens in each category.

In the W breed, IgM and IgG titers binding KLH were both categorized in 3 classes (Table 2.5). Neither IgM nor IgG titers at 20 weeks of age was significantly associated with survival of 20 to 65 weeks of age ($P = 0.17$ and 0.47, respectively).

Table 2.5 Multivariable multilevel logistic analysis of IgM and IgG titers in serum binding KLH at 20 weeks of age, line and body weight at 22 weeks of age (BW22) for survival of 20 to 65 weeks of age in two breeds.

Breed	Variable[1]	class	n	Odds Ratio	95% Confidence Interval	*P*-value
R	IgM titers		408	0.78	0.68-0.91	< 0.01
	IgG titers	< 4.0	55	1.00	Ref	Ref
		4.0-7.9	307	1.40	0.69-2.84	0.35
		≥ 8.0	46	1.24	0.47-3.26	0.66
	Line	B1	68	1.00	Ref	Ref
		B2	58	0.49	0.19-1.45	0.22
		B3	54	0.32	0.09-1.19	0.09
		BA	71	0.66	0.24-1.88	0.45
		BB	91	1.36	0.04-0.30	0.66
		BE	66	1.25	0.40-3.95	0.70
W	IgM titers	< 5.0	56	1.00	Ref	Ref
		5.0-8.9	278	1.46	0.73-2.91	0.28
		9.0-10.9	37	0.60	0.19-1.82	0.37
	IgG titers	< 4.0	84	1.00	Reference	Ref
		4.0-7.9	264	1.29	0.66-2.53	0.45
		≥ 8.0	23	0.75	0.26-2.14	0.59
	Line	W1	79	1.00	Ref	Ref
		WA	37	0.32	0.11-0.94	0.04
		WB	67	0.99	0.33-2.94	0.99
		WC	82	1.60	0.60-4.27	0.35
		WD	40	0.74	0.29-1.89	0.53
		WF	66	0.41	0.09-1.75	0.23
	BW22(g)		371	1.00	0.999-1.003	0.38

[1]R breed = Rhode Island Red; 25.1% of all unexplained variation in the R breed is due to cage; W breed = White Leghorn; 23.0% of all unexplained variation in the W breed is due to cage.

With IgM titers < 5.0 and IgG titers < 4.0 reference classes, the odds ratios for extremely higher IgM titers group (9.0-10.9) and IgG titers group (≥ 8.0) were smaller than 1.00. But for the median groups of both isotype, the odds ratios were larger than 1.00. However, neither of these associations was statistically significant ($P > 0.05$). Line showed a significant effect ($P < 0.01$). Body weight was a confounder of line WA and left in the final model which fitted well (Chi-square = 7.0, $P = 0.54$). 23.0% of all unexplained variation in the survival was due to cage.

Logistic regression analysis was also carried out to study the relationship between the isotypes titers at 40 weeks of age and survival of 40 to 65 weeks of age in both breeds. Results revealed that the probability to survive this laying period was not significantly associated with the titers of isotypes at 40 weeks of age in both breeds. Line was a significant effect in the W breed (data not shown).

2.4 Discussion

In the present study we evaluated the relationships between serum titers of NAb isotypes IgM and IgG binding KLH and survival of a laying period in the White Leghorn and Rhode Island Red layers. As covariates, lines and body weights of the animals at 22 and 40 weeks of age were also evaluated.

2.4.1 Dynamics of NAb isotypes binding KLH in purebred laying hens

NAb in general are believed to remain at tightly regulated levels. Nevertheless, a variety of factors are postulated to modulate B-1 cell functions, and thus NAb secretion, but in a non-cognate manner (Chou et al., 2008). Titers of total NAb in plasma or serum of poultry was found to increase with age (Parmentier et al., 2004; Star et al., 2007), which corresponds with the idea that exogenous stimulation enhances the formation of NAb (Prokesova et al., 1996), as is true for the enhancement of NAb titers by dietary probiotics (Haghighi et al., 2006). However, in mice IgM was found largely unaffected by external antigen (Haury et al., 1997). In mammals, numbers of producing B-1 cells increase from neonatal period to adolescence, but may decrease at later age. Increased susceptibility to infection of the elderly may be partly due to the decrease in NAb (Kohler et al., 2003). In healthy humans and patients with Werner syndrome, titers of IgM NAb may decline with age (Goto et al., 1982), whereas natural IgG titers were found significantly lower in the elderly than in younger adults (Simell et al., 2008). In the present study, a decrease of both NAb isotypes binding KLH in serum with aging (from 20, 40 to 65 weeks) was found. Especially, an abrupt decrease of IgM or IgG titers from 20 to 40 weeks was observed. In 9 of 12 lines, except for WD, BE and B3 from 40 to 65 weeks of age, IgM titers decreased with increasing age. In 8 of the 9 lines, except for line WB, IgM titers decreased significantly from 20 to 40 weeks of age, but only 4 lines decreased significantly from 40 to 65 weeks of age. In 9 of 12 lines, except for WA and WF from 20 to 40 weeks of age, and for W1 from 40 to 65 weeks of age, IgG titers binding KLH decreased with aging. In 8 of these 9 lines, except for line BE, IgG titers binding KLH decreased significantly from 20 to 40 weeks of age, but only 3 lines decreased significantly from 40 to 65 weeks of age (Figure 2.2). Because of the long interval between the three sampling periods, it is not possible in our study to find out if there is an increase with advancing age between 20 and 40 weeks of age, or if there is a peak between age 20 and 40 weeks, followed by a gradual decline. Within lines, both for IgM and IgG, the correlations between titers at 40 and 65 weeks were stronger than that between

20 and 40 weeks, whereas correlations between 20 and 65 weeks were the weakest (Table 2.2 and Table 2.3). The different correlations between different ages suggested that the first 20 weeks, i.e. when birds reach sexual maturity, represents a period of maturation and differentiation of the immune system, followed by a more stable period until 65 weeks of age.

2.4.2 NAb isotype IgM and IgG as predictors for survival in purebred laying hens

The presence and functions of NAb in chicken sera have been reported previously. Non-immunized, normal chickens have pre-existing circulating NAb to various exogenous antigens (Parmentier et al., 2004), self-antigens (Jalkanen et al., 1983; Neu et al., 1984; Barua and Yoshimura, 2001; Parmentier et al., 2004) and (self-) tissue antigens (Bergstra et al., 2010). In part, NAb also regulate specific immunity in poultry (Lammers et al., 2004).

Enhanced survival during a laying period of layers was related with higher titers of total NAb binding KLH, but not with titers of SpAbs (Star et al., 2007). It was proposed that titers of total NAb reflected the individual's capacity to mount an appropriate titer of natural immune defense. In the present study, both natural IgM and IgG binding KLH were detected in the sera. Based on the limited data set, it was not possible to draw conclusions within each line for IgM and IgG NAb isotypes in relation to survival. So the logistic regression analysis was carried out within breed. In both breeds, the lower titers of NAb isotypes especially IgM at 20 weeks of age were observed in the layers which did not survive this period (Figure 2.3). Multivariable multilevel logistic regression analysis indicated that serum titers of IgM and IgG binding KLH at 20 weeks of age were significantly related to the probability to survive the first period of lay (20 to 40 weeks of age) (Figure 2.4 and Table 2.4). If NAb IgM binding KLH at 20 weeks of age increased with one-unit titer, the relative change in risk to die during the first period of lay decreased with 44% in the R breed (odds ratio = 0.56, $P < 0.001$) and decreased 26% in W breed (odds ratio = 0.74, $P < 0.01$). In the R breed, serum titers of IgM (odds ratio = 0.78, $P < 0.01$) at 20 weeks of age was significantly related to the probability to survive the laying period (20 to 65 weeks of age) (Figure 2.5 and Table 2.5). These results suggest that the titer of natural IgM binding KLH in serum is a protective factor enabling surviving, and thus could be indicative for survival. The high significance of IgM is noteworthy, since recently the importance of natural IgM as scavenger, protector and regulator of physiological and immune homeostasis has been highlighted and reviewed (Ehrenstein and Notley, 2010). In mammals and aves, the

IgM isotype is the first antibody to be produced during an immune response, the first to appear during ontogeny, and it is also the oldest, being the sole class of antibody present in all vertebrate species (Fellah et al., 1992). These findings indicate the important evolutionary role of IgM in the immune system. It has been proposed that natural IgM production is driven by endogenous antigen. The diverse functions of natural IgM have become increasingly clear by studying knockout mice that lack natural IgM (Boes et al., 1998a) instead of being regarded as random noise in the immune system. The poly-reactivity and its capacity to facilitate the removal of apoptotic cells entitle natural IgM to participate in seemingly diverse pathophysiologies, including infection, B cell homeostasis, inflammation, atherosclerosis and autoimmunity (Ehrenstein and Notley, 2010).

In addition, IgG binding KLH at 20 weeks of age was also validated to be a protective factor for survival of the first laying period. In the R breed, an odds ratio of 0.72 ($P = 0.02$) for the survival of 20 to 40 weeks was estimated. The existence of natural IgG was reported decades ago. But fewer studies were devoted to the function so far, because of non-predominant character of IgG in the NAb repertoire (Boes, 2000). Plasma cells producing IgG are derived from isotype switched B cells, indicating that a relation of IgM and IgG is not unlikely. In this study, moderate correlations between IgG and IgM were observed (data not shown). Although the odds ratio showed that the titer of IgG was a less sensitive predictor than IgM for survival, the function of natural IgG is still worthwhile for further study.

Average titers of IgM and IgG binding KLH at 40 weeks of age were not significantly related to the probability to survive the second laying period (40 to 65 weeks of age) (data not shown) and thus not as predictive as isotypes titers at 20 weeks of age.

To get proper estimates for isotypes, line, bodyweight, interaction terms and cage were also analyzed when building the model within breed. For the R breed, line was not a significant effect, but kept as a confounder in the model. On the contrary, line was determined as a significant effect in the model for the W breed. This indicated that the chance to survive for the R breed layers was not significantly associated with the genetic origin of the animal. While for W breed layers, the association was significant. This might result from the fact that there was more variation in some survival-related aspects between lines in W breed layers than in R breed layers. In other words, the lines within the W breed were genetically diverse in mortality. The birds from the present W breed in general showed more fear response and feather pecking behavior which may induce death than birds from

the present R breed (Uitdehaag et al., 2008). Furthermore, the lines within the W breed showed varying levels of feather pecking. The line WF was characterized as a high-feather-pecking line in earlier experiments (Rodenburg et al., 2003), and line WB was a more gentle line. In this study, the cause of death was not investigated. We speculate that the R breed layers may mostly die of individual health-related causes, while W breed layers may die of both health-related causes and social interaction causes, such as feather pecking. That may explain why line was a significant effect for survival in the W breed, but not in the R breed. This may also explain why isotype titers were more sensitive and acute parameters for survival in the R breed.

Sustaining immune defense is usually regarded as costly in terms of energy and nutrients. Trade-offs between immune function and other energy-demanding traits are widespread (Mills et al., 2010). Therefore, we also studied bodyweight as a covariate, i.e. whether a trade-off could exist between maintaining NAb isotype titers and body growth, making body weight a potential factor or confounder for survival. Logistic regression analysis indicated that body weight of the layers was neither a significant effect nor an important confounder in the R breed. In the W breed, in the model that verified the relationship between isotypes at 20 weeks of age and survival of 20 to 40 weeks of age, body weight at 22 weeks was an important factor (P = 0.03). However, an odds ratio of 1.00 (95% confidence interval: 1.00-1.01) was estimated, meaning the risk to die changes very slightly due to body weight change.

2.4.3 NAb as predictor of survival to be implemented into the layer breeding program

The population in this study consisted of 12 lines from two breeds. Highly standardized experimental conditions were used: all birds were of the same age, gender, housed in the same facility and provided with the same diets. However, large variations in IgM and IgG titers among breeds, lines, and within lines among individuals were observed. These differences demonstrate that the humoral innate immunity is genetically variable. A major ongoing challenge is to find out how the variations in humoral innate immunity relate to variation in survival and how to utilize this variation (Calder, 2007). We demonstrated a relationship between immune parameters i.e. the NAb isotypes binding KLH and survival in layers. This outcome emphasizes the importance of NAb isotypes and the effect on the animal's survival, especially the survival of 20 to 40 weeks of age, when the peak of egg-laying is also observed. Since the NAb isotype titers can be easily determined in

serum from young layers (20 weeks), routine screening of layers is possible. Layer lines divergently selected for SpAbs responses show different titers of NAb (Parmentier et al., 2004). Heritability of total NAb titers in layer lines was estimated at 0.23 (Wijga et al., 2009), while the heritability of SpAbs was 0.17, suggesting that it is feasible and more efficient to breed on higher NAb isotypes titers to improve the survival of the population.

In conclusion, NAb isotypes IgM and IgG reactive to the foreign antigen KLH could be detected in serum from various layer lines. Significant lower titers of NAb isotypes especially IgM binding KLH at 20 weeks of age were observed in the non-surviving individuals. Serum natural IgM titers at young age could therefore be a good indicator for the ability to survive a laying period. To our knowledge, this is the first study indicating a relationship between distinct NAb isotypes and survival in aves, highlighting the role of IgM. Recently, titers of natural auto-antinuclear antibodies (ANAs) were verified positively related with survival, and negatively related with reproduction in a mammal (Graham et al., 2010). Whether titers of IgM or IgG directed to other (auto-)antigens than KLH are related to survival and reproduction capacity of layers, like in mammals, is subject of current studies.

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3

Genetic parameters and across-line SNP associations differ for natural antibody isotypes IgM and IgG in laying hens

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Abstract

In an earlier study, serum levels of natural antibody (NAb) isotypes IgM and IgG binding keyhole limpet haemopcyanin (KLH) were found to be indicative for survival through the laying period of hens, and therefore considered as promising traits for future implementation in breeding programs for higher survival of layers. In the present study, we first estimated the genetic parameters for the two isotypes at 20, 40, and 65 weeks of age (IgM20, IgM40, and IgM65; IgG20, IgG40, and IgG65). Pooled genetic parameters were estimated from the total population of 2,504 hens from nine purebred layer lines, with line included in the model to account for admixture. Moderate heritabilities (0.14 - 0.44) indicated that selection for isotype titers is feasible, especially for IgM. Secondly, associations between 1,022 single nucleotide polymorphism (SNP) markers and the above-mentioned six immunological traits were estimated in 650 genotyped hens from the nine lines. The association study was performed across lines to detect markers that are closer to the QTL and have the same phase of association in the entire population. Forty-three significant associations between SNPs and isotype titers were detected. The SNPs of interleukins (IL) *IL10* and *IL19* were associated with both isotypes; SNPs of tripartite motif containing 33 (*TRIM33*) and *IL6* showed significant association with IgG20 and IgM20, respectively; SNPs of heat shock protein 90kDa alpha (cytosolic), class B member 1 (*HSP90AB1*) was associated with IgG titers at older ages. Some detected SNPs were also reported associated with other immune and behavioral traits.

Key words: heritability, genetic correlation, across-line association study, single nucleotide polymorphisms, natural antibody, isotype, laying hens

3.1 Introduction

Mortality due to diseases causes substantial economic losses to animal farming, according to the statistics of FAO: in the poultry industry, this loss is estimated to be about 10 to 20% of the gross production value (http://www.fao.org/ag/againfo/themes/en/poultry/animal_health.html). Immune system is the most prominent line of defense against diseases. Compared with environmental factors like management and nutrition, genetic improvement of immunity has permanent and cumulative effects in maintaining health and improving the survival of farm animals. The immune system is complex with numerous components. Identifying measurable traits that characterize an individual's immune system and having better understanding of its genetic background is important to improve immunity through selection.

The immune system is composed of an innate and an adaptive part, which play different roles. Adaptive immunity has proven to be of considerable practical importance, as witnessed by the extensive use of vaccines in animal farming. The adaptive immune response to some specific pathogens or vaccines has been considered in selection for disease resistance and survival (Cavero et al., 2009). The mechanisms as well as the genetic background of adaptive immunity have been well studied. To date, 411 QTL have been reported to be associated with health or disease related traits in chicken (http://www.animalgenome.org/cgi-bin/QTLdb/GG/index), of which most are QTL associated with adaptive immunity. Examples include QTL affecting the resistance (Heifetz et al., 2009) and susceptibility (Heifetz et al., 2007) to Marek's disease in layers, and QTL for resistance to Salmonella in broilers (Ghebremicael et al., 2008). However, the cost of pathogen-challenge trials for these studies is very high and presents biosecurity risks. Furthermore, as these QTL are related with some specific disease, it is difficult to make a decision about which ones should be considered in a genetic selection program (Lamont 1998).

The innate immune system is ready to act and can stop infections before they cause disease. Natural antibodies (NAb), which are antibodies present in the circulatory system in the absence of a deliberate antigen exposure, are an important humoral part of innate immunity (Avrameas 1991). An increasing amount of evidence indicates that NAbs have broad reactivity against foreign antigens and play multiple roles in health and disease. NAbs link innate and adaptive immunity (Ochsenbein and Zinkernagel 2000); prevent pathogen dissemination to vital organs and improve immunogenicity through enhanced

antigen-trapping in secondary lymphoid organs (Ochsenbein et al., 1999); maintain the tissue homeostasis by suppressing the development of inflammation (Anania et al., 2010). Star et al. (2007) found a relationship between total NAb levels and survival in laying hens. QTL for total NAb titers and classical and alternative complement activity were recently reported (Biscarini et al., 2010a). Natural, as well as acquired, antibodies comprise different immunoglobulin (Ig) classes known as isotypes: IgM, IgG (IgY), and IgA (in avian species). Evolution of the various Ig classes has made a significant contribution to functional diversity in terms of antigen processing and recruitment of effector mechanisms (Janeway et al., 2001). A previous study in laying hens indicated that NAb isotype titers -especially IgM at 20 weeks of age- are significantly associated with survival through the laying period, and could be used as more accurate predictors of survival (Sun et al., 2011) compared with total NAbs titers (Star et al., 2007). QTL for -or markers associated with- NAb isotypes IgM and IgG have however not been reported yet.

In the present study the genetic background of NAb isotypes IgM and IgG in laying hens was investigated. First, heritability and genetic correlations for IgM and IgG were estimated. Then an association study using 1,022 SNP markers was performed to identify possible QTL or candidate genes for NAb isotypes. A population of laying hens from nine commercial purebred lines was used in the analysis, adopting an across-line approach with testing of SNP-by-line interaction. To our knowledge, this is the first study to estimate genetic parameters and to perform an association study for distinct NAb isotypes (IgM and IgG) in poultry. The results will help to better understand the genetic control of levels of innate immunity, thus disclosing opportunities to breed for higher survival in laying hens.

3.2 Materials and methods

In a previous study, Biscarini et al. (2010a) used the same genotype data as in the current study to detect QTL for total NAbs titers. Sun et al. (2011) measured the NAbs isotype titers (IgM and IgG) and related them to survival through a laying period. In the current study, we aim at estimating the genetic parameters and detecting the associated chromosomal regions for NAbs isotypes levels.

3.2.1 Study population

The animal population used in the genetic parameters study consisted of 2,504 hens from nine commercial purebred layer lines from the Institut de Sélection Animale (ISA) B.V., a Hendrix Genetics company (The Netherlands): four Rhode

Island Red lines (B1, B2, B3, and BB) and five White Leghorn lines (W1, WA, WB, WC, and WF). These lines were not under direct selection for the traits of concern in the current study. The animals were recorded progeny of 181 sire and 375 dams. Every rooster was mated on average with 2-3 females, while each hen was mated with only one male. These animals had unique barcode wing bands assigned at one day old, allowing for identification of individuals. For the association study, 650 layers from the nine lines were included. The pedigree of the layers was provided by ISA and consisted of 267,118 animals. The laying hens were observed over the whole laying period, from 17 until 72 weeks of age. Housing, feed and vaccinations were described in Star et al. (2007). Blood samples of the hens were collected at 20, 40, and 65 weeks of age by wing vein puncture for extracting serum and measuring immune traits. These time points represent birds close to sexual maturity (20 wk), middle age (40 wk) and birds close to the end of the laying period (65 wk). The isotype titers at these ages may convey different information. The blood sample at 40 weeks of age was also used for extracting DNA for genotyping.

3.2.2 Phenotypes

NAb isotypes IgM and IgG titers binding KLH at 20, 40, and 65 weeks of age (IgM20, IgM40, and IgM65; IgG20, IgG40, and IgG65) in the serum sample were measured using indirect enzyme-linked immunosorbent assay (ELISA) as described by Sun et al. (2011). The number of available observations at different ages and descriptive statistics are shown in Table 3.1. For some animals, it was not possible to determine isotype titers at each age, since their samples were exhausted when used previously for other analysis.

3.2.3 Genotype

Genotyping was carried out in a 1,536-plex format using the GoldenGate assay (Illumina, San Diego) by a commercial genotyping facility (Service XS, Leiden, The Netherlands). The 1,536 SNPs were selected to cover some immune and behaviour related-QTL regions as well as candidate genes on 24 of the 39 chicken chromosomes. Positions of the SNPs were derived from the NCBI database (Galgal 2.1 build 128). In total, the population was successfully genotyped for 1,356 SNPs. SNPs deviating from Hardy-Weinberg equilibrium at the Bonferroni-corrected 0.05 significant threshold, non-segregating SNPs over all lines and SNPs with a minor allele frequency ≤ 0.05 were discarded from the analysis, resulting in 1,022 SNPs for association analysis (Table 3.2). Further details on SNP screening can be found in Biscarini et al. (2010a).

Table 3.1 Number of layer hens in each line used in the study and mean and standard deviation of traits for the single lines and the overall population.

		n			IgM binding KLH[1]			IgG binding KLH[1]		
Breed[2]	Line	20wk	40wk	65wk	IgM20	IgM40	IgM65	IgG20	IgG40	IgG65
R	B1	60	66	7	8.21	6.82	6.81	7.13	5.33	5.21
	B2	57	69	80	7.42	6.71	6.47	6.42	5.05	4.78
	B3	48	61	71	8.01	6.01	6.58	6.59	4.54	4.05
	BB	63	57	63	7.11	5.55	5.35	5.97	4.20	3.22
W	W1	57	58	70	8.48	7.20	6.75	5.88	5.21	5.30
	WA	69	63	64	6.03	4.53	4.18	4.60	5.03	3.87
	WB	22	45	51	4.53	4.71	4.61	4.28	3.87	2.69
	WC	49	65	67	7.62	5.74	4.63	6.60	4.48	3.73
	WF	46	49	57	8.37	7.37	6.59	4.47	5.43	4.36
Total		471	533	600						
mean					7.43	6.09	5.84	5.86	4.82	4.21
SD[3]					1.68	1.43	1.54	1.80	1.49	1.66
C.V.(%)[4]					22.6	23.5	26.4	30.7	30.9	39.4

[1] IgM20, IgM40, IgM65 = natural antibody isotype IgM titers binding keyhole limpet hemocyanin (KLH) at 20, 40, and 65 wk of age; IgG20, IgG40, IgG65 = natural antibody isotype IgG titers binding KLH at 20, 40, and 65 wk of age.
[2] R = Rhode Island Red, W = White Leghorn
[3] SD = standard deviation
[4] C.V. (%) = percentage coefficient of variation

3.2.4 Genetic parameters estimation

Heritabilities, genetic and phenotypic correlations were estimated for all six traits (IgM20, IgM40, and IgM65; IgG20, IgG40, and IgG65) based on the entire available population, including hens whose phenotype was measured but were not genotyped. There were 2166 animals for IgG20 and IgM20, 2,175 for IgG40 and IgM40, and 851 for IgG65 and IgM65, with proportionally similar sample size for each line. Heritabilities were estimated by restricted maximum likelihood (REML) with a univariate model:

$$Y_{ij} = \mu + Line_i + a_j + e_{ij} \quad (1),$$

Where Y_{ij} is the observation of any given trait on animal *j* of line *i*, μ is the overall mean, $Line_j$ is the fixed effect of genetic line (nine classes); a_j is the random additive genetic effect of the animal *j*; $\mathrm{var}(a) = A\sigma_a^2$, with *A* being the additive genetic relationship matrix; e_{ij} is the residual term with $\mathrm{var}(e) = I\sigma_e^2$. Heritabilities were calculated as $h^2 = \sigma_a^2 / (\sigma_a^2 + \sigma_e^2)$. Genetic and phenotypic correlations between traits were estimated based on bivariate analyses using model (1).

Table 3.2 Total numbers of SNPs per chromosome, numbers of fixed SNPs and number of SNPs with a minor allele frequency (MAF) ≤ 0.05 across-line.

Chromosome	Size (Mbp)[1]	All SNPs	Used SNPs	Fixed	SNPs with MAF ≤ 0.05
GGA1	201	68	61	11	4
GGA2	155	27	23	5	5
GGA3	114	140	121	12	11
GGA4	94	422	371	67	34
GGA5	62	285	265	26	17
GGA6	37	27	22	1	3
GGA7	38	175	149	18	14
GGA8	31	7	7	1	0
GGA9	26	12	10	0	1
GGA10	22.6	4	3	0	0
GGA11	21.9	8	6	3	1
GGA12	20.5	7	7	2	1
GGA13	18.9	37	32	3	2
GGA14	15.8	7	6	0	0
GGA15	13	7	5	1	1
GGA16	0.43	22	16	3	0
GGA17	11.2	7	7	0	1
GGA19	9.9	27	20	4	1
GGA21	7	4	4	1	0
GGA22	3.9	3	2	0	0
GGA23	6	4	2	1	0
GGA24	6.4	18	17	3	1
GGA26	5.1	59	50	7	4
GGAZ	75	158	135	38	11
Total		1,536	1,341	207	112

[1] Size: size of the whole chromosome as derived from the NCBI chicken genome database.
[2] unmapped SNP.

3.2.5 Association study

For the association study, phenotypes and genotypes were available for 471 to 600 individuals, depending on the trait. The number of observations per line ranged from 22 at 20 weeks of age (line WB) to 80 at 65 weeks of age (line B2), with an average of 59 (Table 3.1). A two-step single SNP across-line association study, as described in Biscarini et al. (2010a) was performed. First, each SNP was fitted individually as a fixed effect in a general linear model, without accounting for the genetic relationship between the animals. Afterwards, the potentially interesting SNPs detected in the first step were verified in a mixed model, taking additive genetic relationships into account.

In the first step, single SNP analyses were performed across lines. The statistical model was:

$$Y_{ijk} = \mu + SNP_i + Line_j + (SNP \times Line)_{ij} + e_{ijk} \quad (2),$$

Where Y_{ijk} was the observation of any given trait on animal *k*, μ is the overall mean, SNP_i was the fixed effect of SNP genotype at locus *i* (either AA, AB or BB), $Line_j$ was the fixed effect of line (nine classes), $(SNP \times Line)_{ij}$ was the interaction between SNP genotype and line, and e_{ijk} was the random residual. Line effect was included in the model to account for the genetic difference between lines. There were few (1 to 3) animals with missing genotypes for a limited number of SNPs, whose records were not included in the analysis. This model was run twice, once without the $(SNP \times Line)_{ij}$ term to obtain significance level for the SNP effect and once with the $(SNP \times Line)_{ij}$ term to determine the significance level for the interaction. SNPs that had a significant effect ($P \leq 0.05$) but did not show a significant SNP-by-line interaction ($P > 0.05$) were considered to show significance across lines. The significant SNPs that met at least one of the following criteria were selected for the second step in which genetic relationships among the animals were accounted for. The selection criteria for the SNPs were:

1. Significant SNP effect for single trait with $P \leq 0.001$ and no SNP-by-line interaction;
2. Significant SNP effect ($P \leq 0.05$) consistent for the same isotype (IgM or IgG) over time (2 or 3 time points) and no SNP-by-line interaction;
3. Significant SNP effect ($P \leq 0.05$) consistent for both IgM and IgG at the same time point and no SNP-by-line interaction;

Quantitative traits may also be influenced by background genes. Accounting for genetic relationships among the animals in an association study is more appropriate (Kennedy et al., 1992), since it helps avoiding spurious associations. In the second step, a polygenic effect was added to the model to account for family relationship among animals within lines. SNPs selected in the first step were analysed using a mixed animal model:

$$Y_{ijk} = \mu + SNP_i + Line_j + a_k + e_{ijk} \quad (3),$$

where all the terms were as in model (2) except a_k, which is the random genetic effect of the *k*th animal. $\mathrm{var}(a) = G = A\sigma_a^2$, where *A* is the additive genetic relationship matrix, and $\mathrm{var}(e) = R = I\sigma_e^2$. The ratio between residual and genetic variances $(\sigma_e^2 / \sigma_a^2)$ was fixed for each trait using the previously estimated heritabilities. The additive genetic relationship matrix was based on four-generations of ancestors extracted from the pedigree file provided by ISA. The SNPs that still showed a significant effect ($P \leq 0.05$) on the traits from model (3) were considered as a genomic region of interest.

Genetic parameters with model (1), polygenic effects and SNP effects as described in model (3) were estimated with a REML procedure in ASReml (Gilmour et al., 2006). The open source programming environment R was used for data editing, descriptive statistics, and association analysis with model (2).

3.3 Results

3.3.1 Descriptive statistics of the traits

Descriptive statistics of the six traits (IgM20, IgM40, and IgM65; IgG20, IgG40, and IgG65) are summarized in Table 3.1. In general, IgM and IgG titers decreased with age (20, 40, and 65 weeks of age) in most of the nine lines, except for IgM in line B3 and WB, and IgG in line W1, WA and WF. Especially, an abrupt decrease of IgM or IgG titers from 20 to 40 wk was observed. The across-line coefficient of variation (C.V.) ranged from 22.6% (IgM20) to 39.4% (IgG65), indicating substantial variation associated with these immunological traits in hens. On average, the C.V. for IgM was lower than that for IgG. There were substantial differences in isotype titers between lines: e.g. for IgG20 titers ranged from 4.28 (line WB) to 7.13 (line B1).

3.3.2 Genetic parameters of the traits

Estimates for heritabilities (h^2), genetic (r_g) and phenotypic (r_p) correlations for NAb isotypes are presented in Table 3.3. Heritability estimates ranged from $h^2 = 0.14$ for IgG40 to $h^2 = 0.44$ for IgM65. The heritabilities for IgM titers at different ages were around 0.4, while for IgG they varied from 0.14 for IgG40 to 0.31 for IgG20. IgM titres were more heritable than IgG at each age.

Phenotypic and genetic correlations between traits were positive. Phenotypic correlations between the same isotype titers at different ages were generally moderate, but higher between 40 and 65 weeks of age: $r_p = 0.63$ for IgG40 and IgG65 and $r_p = 0.59$ for IgM40 and IgM65. Phenotypic correlations between IgM and IgG at the same age were around 0.25. In general, genetic correlations between titres of the same isotype at different ages were quite high, especially between IgM40 and IgM65 ($r_g = 0.95$) and between IgG40 and IgG65 ($r_g = 0.99$). Genetic correlations between IgM and IgG titres at the same age were around 0.2.

Table 3.3 Estimates of heritabilities (bold, on the diagonal), genetic correlations (above the diagonal) and phenotypic correlations (below the diagonal) for the traits. Associated standard errors (SE) are shown in the parenthesis.

Trait[1]	IgM20	IgM40	IgM65	IgG20	IgG40	IgG65
IgM20	**0.41(0.05)**	0.57(0.08)	0.81(0.11)	0.24(0.10)	0.08(0.15)	0.52(0.19)
IgM40	0.39(0.02)	**0.42(0.05)**	0.95(0.07)	0.02(0.11)	0.23(0.14)	0.35(0.17)
IgM65	0.32(0.04)	0.59(0.02)	**0.44(0.09)**	0.36(0.20)	0.07(0.20)	0.24(0.17)
IgG20	0.30(0.02)	0.04(0.02)	0.08(0.04)	**0.31(0.05)**	0.39(0.15)	0.78(0.20)
IgG40	0.10(0.02)	0.24(0.02)	0.13(0.04)	0.36(0.02)	**0.14(0.04)**	0.99(0.12)
IgG65	0.14(0.04)	0.13(0.04)	0.29(0.03)	0.28(0.04)	0.63(0.02)	**0.26(0.09)**

[1] IgM20, IgM40, IgM65 = natural antibody isotype IgM titers binding keyhole limpet hemocyanin (KLH) at 20, 40, and 65 wk of age; IgG20, IgG40, IgG65 = natural antibody isotype IgG titers binding KLH at 20, 40, and 65 wk of age.

3.3.3 Association study

In the first step of the association study, 1,022 SNPs were analyzed across-line with model (2). There were 302 SNPs that showed significant association ($P \leq 0.05$) with at least one of the traits, of which 275 did not show a significant SNP-by-line interaction ($P > 0.05$). After applying the established criteria, 57 of these 275 SNPs were selected for the next step of analysis where family structure was taken into account. Eventually, 43 SNPs still showed significant association (Table 3.4). The false discover rate (FDR) was calculated based on the *P*-values from model (2) for

association between 1,022 SNPs and each trait. The FDR for the SNPs presented in Table 3.4 ranged from 0.005 to 0.74, and 25% of the significant associations had a FDR < 0.20. Seventy-five percent of the significant SNPs identified in model (2) were confirmed with model (3) (43 out of 57), especially the highly significant ones. The Pearson's correlation coefficient between -Log_{10} (*P*-value) of all significant associations with and without the polygenic effect was 0.77.

The detected association comprised 12 SNPs for IgG20, 13 for IgG40, 10 for IgG65, 12 for IgM20, 7 for IgM40, and 10 for IgM65. Twenty-two of these SNPs were associated with only one trait; the other 21 SNPs were associated with two traits. The strongest association was found for IgM40 (-Log_{10} (*P*-value) = 4.46) on GGA3. Based on the significant SNP associations, potential QTL for NAb isotypes in laying hens have been found. Examples include QTL for IgG20 on GGA3 (SNPs rs13503408 and rs13503482) and GGAZ (SNPs rs16105367, rs16105275, rs16105159 and rs16105051); QTL for the IgM20 on GGA4 (SNPs rs13514674 and rs13514692) and GGA5 (SNPs rs15660844 and rs15661104). On GGA3, a potential QTL for IgM40 and IgM65 was detected at SNP rs13717504.

3.4 Discussion

3.4.1 Genetic parameters

In the present study, we estimated genetic parameters for NAb isotypes IgM and IgG binding KLH at different ages based on the whole available population of laying hens (nine purebred lines), including the animals with isotype titers but that were not genotyped. Layers from different lines were not related based on the relationships registered in the pedigree files. Line is a known source of NAb isotype variation (Sun et al., 2011). Fitting 'line' as a random effect in the model allows for the estimation of between-line variation. The results showed that there was a substantial contribution of the between-line variation to the total phenotypic variance: from 17% for IgG20 to 42% for IgM20. For IgM this proportion was approximately twice as large as for IgG at the same age, which indicated that the variation of IgM NAb titres between lines was larger than that of IgG. In the present study, we included line as a fixed effect in model (1) to account for the between-line variation and to avoid biased heritability estimates for the two different isotypes.

The heritabilities for IgM titres at different ages were all around 0.4. For IgG, the heritabilities varied at different ages, ranging from 0.14 at 40 weeks of age to 0.31

Table 3.4 SNPs significantly associated with natural antibody isotype IgM and IgG titers binding KLH at 20, 40, and 65 wk of age. -Log_{10} (*P*-value) are reported in the columns.

Chromosome	SNP	kbps	cM[1]	IgG20[2]	IgG40	IgG65	IgM20	IgM40	IgM65	Candidate gene[3]	Immune/Behaviour-related traits[4]
GGA2	rs15937157	30892798	77.2				1.32			*IL6*	
GGA3	rs13775191	3920339	9.8	3.23	1.42						
GGA3	rs13773711	5093724	12.7		1.43	1.64					
GGA3	rs13504786	7469634	18.7		1.44	1.80					
GGA3	rs13504160	8503787	21.3					2.22	2.30		
GGA3	rs13503482	9694915	24.2	2.32						*AFTPH*	BR51s
GGA3	rs13503408	9866493	24.7	3.00							BRBC20, BR51s
GGA3	rs13717504	19407727	48.5					4.46	1.46		Belly51s
GGA3	rs16246580	31512500	78.8		2.70	1.92				*HSP90AB1*	
GGA4	rs13578397	30698644	76.7				1.36			*MAML3*	
GGA4	rs13512983	30896422	77.2			1.55			2.52		BR69s
GGA4	rs13514531	34556210	86.4		1.46						
GGA4	rs13514674	34725975	86.8				1.68				
GGA4	rs13514692	34779481	86.9		1.64		1.43				
GGA4	rs13516409	39982138	100.0	3.00						*NPNT*	
GGA4	rs13518664	46910110	117.3			2.52				*BMP3*	

Table 3.4 (continued) SNPs significantly associated with natural antibody isotype IgM and IgG titers binding KLH at 20, 40, and 65 wk of age. -Log_{10} (*P*-value) are reported in the columns

Chromosome	SNP	kbps	cM[1]	IgG20[2]	IgG40	IgG65	IgM20	IgM40	IgM65	Candidate gene[3]	Immune/Behaviour related traits[4]
GGA4	rs13519142	47791554	119.5					1.64			
GGA4	rs13522073	54208891	135.5		1.96						
GGA4	rs16422070	62651897	156.6	1.48			1.33				Belly51s
GGA5	rs14513071	10513039	26.3		1.60			2.15			
GGA5	rs15658688	10635972	26.6				2.10		1.96	*ST5*	
GGA5	rs15660844	11445142	28.6				1.60		1.55	*PDE3B*	
GGA5	rs15661104	11551652	28.9				1.64				
GGA5	rs15668593	15358239	38.4				1.38				
GGA5	rs15674192	18427037	46.1		1.35	1.44					
GGA5	rs13586877	40989063	102.5						1.72	*Open Reading Frame*	
GGA6	rs15806042	27691999	69.2			1.89				*ADRA2A*	
GGA7	rs13596168	25491840	63.7	3.00							Belly69s
GGA7	rs13600494	35478765	88.7	1.30			1.43				
GGA7	rs13600581	35696491	89.2		1.40						
GGA13	rs14064896	17451586	43.6					1.32	1.92	*IRF1*	NCD20, SRBC20, SRBC65, BRBC65, LPS65

Table 3.4 (continued) SNPs significantly associated with natural antibody isotype IgM and IgG titers binding KLH at 20, 40, and 65 wk of age. -Log_{10} (*P*-value) are reported in the columns

Chromosome	SNP	kbps	cM[1]	IgG20[2]	IgG40	IgG65	IgM20	IgM40	IgM65	Candidate gene[3]	Immune/Behaviour related traits[4]
GGA13	rs15709619	17527265	43.8						2.05	*IL13*	Belly51s
GGA26	rs13606001	2062437	5.2				1.46	1.92			
GGA26	rs14298901	2376225	5.9		2.40			2.05		*IL10*	SRBC20, LPS40
GGA26	rs14298911	2385653	6.0		2.70	2.30				*IL19*	
GGA26	rs13606106	2461645	6.2		1.30				1.39		
GGA26	rs13606552	3679472	9.2	1.68						*TRIM33*	
GGAZ	rs16102814	12734147	31.8			1.54	2.40				
GGAZ	rs16105367	23202071	58.0	1.92							
GGAZ	rs16105275	23335626	58.3	1.40						*ANKDD1B*	
GGAZ	rs16105159	23468761	58.7	2.22							Belly51s
GGAZ	rs16105051	23571939	58.9	1.49		2.10				*GCNT4*	
GGAZ	rs13795422	34067107	85.2						2.15		

[1] 1cM = 4×10^5 bp.
[2] IgG20, IgG40, IgG65 = natural antibody isotype IgG titers binding keyhole limpet hemocyanin (KLH) at 20, 40, and 65 wk of age; IgM20, IgM40, IgM65 = natural antibody isotype IgM titers binding KLH at 20, 40, and 65 wk of age.
[3] *IL6* = gene encoding cytokine Interleukin 6; *AFTPH* = gene encoding protein Aftiphilin; *HSP90AB1* = gene encoding Heat shock protein 90kDa alpha (cytosolic), class B member 1; *NPNT*: gene encoding nephronectin, also called *POEM* (preosteoblast EGF repeat protein with MAM domain);

BMP3 = gene encoding bone morphogenetic protein 3; *MAML3* = gene encoding *Gallus gallus* mastermind-like 3 (Drosophila) (predicted); *ST5* = gene encoding suppression of tumorigenicity 5; *PDE3B* = gene encoding phosphodiesterase 3B, cGMP-inhibited; *ADRA2A* = gene encoding adrenergic, alpha-2A-, receptor; *IRF1* = gene encoding interferon regulatory factor 1; *IL13* = gene encoding cytokine Interleukin 13; *TRIM33* = gene encoding tripartite motif containing 33; *IL10* = gene encoding cytokine Interleukin 10; *IL19* = gene encoding cytokine Interleukin 19; *ANKDD1B* = gene encoding ankyrin repeat and death domain containing 1B; *GCNT4*: gene encoding glucosaminyl (N-acetyl) transferase 4, core 2.

[4] BR51s, BR69s = sum of the individual feather scores for the back and rump regions at 51 and 69 wk of age (scale 0-10); Belly51s, Belly69s = individual feather scores for the belly region at 51 and 69 wk of age (scale 0-5); BRBC20, BRBC65 = alternative complement activity at 20 and 65 wk of age; SRBC20, SRBC65 = classical complement activity at 20 and 65 wk of age; NCD20 = acquired antibody titers for the Newcastle disease virus at 20 wk of age; LPS40, LPS65 = natural antibody titers for lipopolysaccharide at 40 and 65 wk of age.

at 20 weeks of age (Table 3.3). This moderate heritability for NAb isotypes indicates that selection for NAb isotype titres is feasible, especially for IgM.

The heritability for specific antibodies (SpAb) against sheep red blood cells (SRBC) was estimated to be 0.31 (Pinard et al., 1992) and 0.18 (Bovenhuis et al., 2002), after divergent selection of layers for high and low antibody response for 9 and 18 generations, respectively. Using these lines, Wijga et al. (2009) estimated a heritability of 0.23 for total NAbs binding rabbit red blood cells. The heritability for SpAb isotypes titres was reported higher than for the total SpAb response to the same antigen: heritability for SpAb isotypes IgM and IgG in response to SRBC stimulation was as high as 0.61 and 0.52, respectively (Sarker et al., 1999). This is the first time the heritabilities for the NAb isotypes IgM and IgG are estimated in poultry, so a direct comparison with literature results is not possible. In cows, IgM NAbs were found to be more heritable than IgG NAbs (Ploegaert et al., 2010), which is in line with our results in layers.

Antibody isotypes differ in molecular structure, biological properties, location of production and their function. IgM antibodies are the most abundant antibodies circulating in the body and act as the first line of defense against a foreign molecule or invading organism. Natural IgM antibodies are polyreactive and participate in diverse physiological processes including response to infection, cell homeostasis, inflammation, atherosclerosis and autoimmunity (Ehrenstein and Notley 2010). Study with the IgM-deficient mice showed that IgMs play a role in protection against the influenza virus (Baumgarth et al., 2000) and bacterial infections (Boes et al., 1998). It is presumed that the production of the IgM isotype is driven by endogenous (auto) antigens and largely unaffected by external antigens (Haury et al., 1997). B cells can change the isotype of the antibody they express (from IgM to IgG or IgA) by isotype class-switch after being activated (Market and Papavasiliou 2003). At the molecular level this process involves the orderly somatic rearrangement of Ig heavy chain constant region genes. It is a highly regulated biological process by T-helper cells and antigen-presenting cells and their cytokines such as interleukin (IL)4, IL5, IL10, and transforming growth factor, beta 1 (TGFB1) (Stavnezer and Amemiya 2004). The observed higher heritabilities for IgM than IgG agree with the observation that IgM NAbs are naturally present regardless of environmental factors, while their transformation into IgG NAbs likely occurs in response to the effect of stimulating factors present on the B cells.

For either IgM or IgG, there are strong and positive genetic correlations between titers at older ages (40 and 65 weeks of age): r_g = 0.95 between IgM40 and IgM65;

r_g = 0.99 between IgG40 and IgG65. This suggests that NAb isotype titres at 40 and 65 weeks of age are genetically the same trait, but differ from NAb isotype titers at 20 weeks of age. Genetic correlations between IgM and IgG at the same age were around 0.2. The moderate positive genetic correlation suggests that IgM and IgG only partially share the same genetic background and are relatively independently controlled.

3.4.2 Methodological aspects of the association study

An across-line SNPs association study for NAb isotypes IgM and IgG titers binding KLH was performed: the main feature of the across-line association study is that markers close to QTL and valid in the whole population (i.e. in all lines) are targeted (Biscarini et al., 2010a). First, each SNP was individually fitted as a fixed effect in a linear model and tested for SNP by line interaction; afterwards, the potentially interesting SNPs detected in the first step were validated accounting for relationships among the animals. Finally, 43 significant SNPs associations with NAb isotypes were detected.

The sample size used for the association study of each trait was different due to randomly exhausted serum samples (Table 3.1). To understand how sample size affects the results of the analysis, we carried out the association study for IgM20 in a randomly selected subset with 90%, 80%, 70%, 60% and 50% of the original population (5 replicates for each subset size). Results indicated that 87%, 77%, 66%, 44%, and 34% of the significant SNPs detected in the original population could be confirmed, respectively. The strongest associations were also prone to be confirmed in smaller samples: SNP rs13596168, for instance, was associated with IgG20 with a significance level of $-\text{Log}_{10}$ (*P*-value) = 3.40 in the original sample and could be detected in three of the five replicates with 50% of the sample size. This indicated the robustness of the model for the analysis. The associations detected in the present study are therefore unlikely to show relevant changes due to the slight differences in sample size, especially the highly significant ones.

3.4.3 Patterns of association

The patterns of association show that closely linked SNP were not always associated with the same isotype at the same age; the associations were not consistent over all 3 ages for the same isotype. Only five SNPs significantly associated with IgG and IgM at the same age were observed (Table 3.4). This was in agreement with the estimated weak genetic correlation between IgG and IgM at

the same age (Table 3.3). SNPs significantly associated with IgG65 tended to be associated also with IgG40 (5 out of 10). A similar pattern was found for IgM: 3 out of 7 of the significant SNPs for IgM40 were associated also with IgM65. This is in agreement with the observation of high genetic correlations between the same isotype titers at older age (Table 3.3) and hypothesis that NAb isotypes titres at 40 and 65 weeks of age are genetically the same trait. The fact that not always the same SNPs were found to be associated with isotype titers at 40 and 65 weeks of age may be interpreted as due to 1) the genetic correlations between the isotypes at successive ages were high, but not as high as 1, and 2) the power of the association study was not equal to 1 implying that even if a SNP has an effect it might not appear to be significant in the present analysis (false negative). In most cases, the same SNP had consistent direction of effect on the isotype titers at both 40 and 65 weeks of age (data not shown). There were fewer SNPs associated with IgM20 (3 out of 11) or IgG20 (2 out of 12) which also were associated with the same isotypes at later ages. The detected association patterns together with the different genetic correlations between different ages, are in line with expectations: NAbs are secreted by B-1 cells which, in mammals, are characterized by the expression of CD5 (Kasaian and Casali 1993). The origin of NAbs in chicken is unknown yet, due to the scarcity of information on chicken B-1 cells, but all chicken B cells express the CD5 marker (Koskinen et al., 1998). The bursa of Fabricius, where the B cells are produced, starts a regression phase from 9 weeks of age onwards, until nearly disappearing when sexual maturity is reached (around 21 weeks of age for laying hens when they start laying eggs). So we speculate that young chickens (20 weeks of age) have not fully develop yet their immune repertoire as compared to older chickens (40 and 65 weeks of age), whose immune status is likely more developed and in equilibrium with the environment. The genetic background for developing a functional innate immunity to produce NAb isotypes is different from that for maintaining a stable isotype production until 65 weeks of age.

3.4.4 Detected associations and candidate genes

There were 26 SNPs significantly associated with IgM (Table 3.4). On GGA2, SNP rs15937157 was situated in *IL6* gene and was associated with IgM20. IL6 is an interleukin family member. The functions of IL6 range from key roles in acute-phase protein induction to stimulation of B cell growth, proliferation and terminal differentiation into mature antibody producing plasma cells (Muraguchi et al., 1988; Tackey et al., 2004). A functional homologue of mammalian IL6 is found in

chickens (Schneider et al., 2001) and involved in the infectious process like arthritis induced by staphylococcus (Zhou et al., 2007). To the best of our knowledge, this is the first time the IL6 was implicated in the regulation of IgM levels. Putative QTL for IgM20 were found on GGA4 (SNPs rs13514674 and rs13514692) and GGA5 (SNPs rs15660844 and rs15661104). There are no known candidate genes in these regions. Earlier we demonstrated that NAb titers -especially the IgM isotype at 20 weeks of age- are predictive of survival through the laying period: odds ratios of 0.56 ($P < 0.001$) for IgM20 were estimated, which means that if IgM20 titers increase by one unit, the relative change in risk of dying during the laying period decreases by 44% (Sun et al., 2011). The detected QTL and SNPs for IgM20 are promising targets for selection for improved survival.

On GGA13, a QTL for IgM40 and IgM65 (SNPs rs15709619 and rs14064896) was suggested. Two candidate genes are found in this region: IL13 and interferon regulatory factor 1 (*IRF1*). IL13 is a cytokine secreted by many cell types, especially T helper type 2 cells, and is a mediator of allergic inflammation and disease (Wynn 2003). Most of the biological effects of IL13 are the same as the closely located IL4, whose production requires NAb isotype IgM (Stager et al., 2003). IL4 can stimulate strong B-1 cell proliferative response (Tsuji et al., 2002). It is possible that the similar regulation mechanism also happens between IgM NAbs and IL13. *IRF1* is in the proximity of *IL4*. Beyond its function as a transcription factor, IRF1 has been shown to play multiple roles in disease/health. It acts as tumor suppressor in breast cancer (Bouker et al., 2005), regulates cell apoptosis in mammary (Bowie et al., 2004), and fights virus infections like West Nile viruses (Brien et al. 2011). In laying hens, *IRF1* was found to be associated with Ascaridia galli worm burden (Luhken et al., 2011). IgM NAb was also reported to have similar functions like IRF1. Possibly IRF1 and IgM are involved in the same mechanism or network.

There were 28 SNPs detected to be significantly associated with IgG (Table 3.4). The gene tripartite motif containing 33 (*TRIM33*) on GGA26 (SNP rs13606552) was found to be associated with IgG20. TRIM33 is involved in the regulation of TGFB1 signal transduction (Moustakas and Heldin 2009) and in cancer suppression in murine (Herquel et al., 2011). The role of TRIM33 in chicken is unknown. However, its association with IgG titers in laying hens suggests further studies on the role of TRIM33 and its relation with IgG NAbs in the immune response in poultry. A QTL for IgG20 (SNPs rs13503408 and rs13503482) was also found on GGA3, in a region with no known candidate genes. On GGAZ, a QTL (SNPs rs16105367, rs16105275, rs16105159 and rs16105051) for IgG20 was detected. Birds have female heterogamety with Z and W sex chromosomes. Hens were reported to have higher

SpAb titers against SRBC than cocks, and the heritability for this trait was also higher in hens (Gross et al., 1980; Boa-Amponsem et al., 1997). This difference might be due to the genes located on the sex chromosome, suggesting that male and female antibody titers are genetically different traits. However, Bovenhuis et al. (2002) estimated a genetic correlation of 0.92 between male and female SpAb titers which is in contrast with this hypothesis. It is also not clear if NAbs isotype titers in male and female are the same traits. In the present study, only hens were examined, therefore the comparison between female and male was not possible.

In the previous study, IgG20 were also identified to be predictive of survival during the laying period: the odds ratio for IgG20 was 0.72 (P = 0.02), indicating a decrease of 28% in the risk of dying per unit increase in IgG (Sun et al., 2011). SNPs which are associated with IgG20 might also be used in selection for high survival. The fact that different SNPs are detected for IgM20 and IgG20 might due to several reasons: e.g. lower genetic correlations and power of association analysis, as we discussed before. Biologically, since formation of antibody isotypes, and isotype switching from IgM to IgG are differentially regulated by various (T-cell derived) cytokines, it is not surprising that different SNPs related with different cytokines or other genes were identified. The positive genetic correlation between IgM20 and IgG20 indicates that there are possibilities to improve both traits simultaneously. However, this does not exclude the presence of chromosomal regions with antagonistic effects on IgM20 and IgG20. Validation and good estimation of the SNP effects is an important step between our results and their practical application in breeding. Further study is indispensable.

On GGA3, SNP rs16246580 was associated with IgG40 and IgG65. Two adjacent SNPs, SNP rs14334093 and rs15312051 were also associated with IgG40. The gene heat shock protein 90kDa alpha (cytosolic), class B member 1 (*HSP90AB1*) which encodes 90-kDa heat shock protein (HSP90) was located nearby. HSP90 is an important phylogenetically conserved structure and immunodominant self-antigen (Pashov et al., 2002). It is widely accepted that the production of NAbs rests on positive selection by self-antigens including heat shock protein (HSP) (Baumgarth et al., 2005). Shinozaki et al. (2006) found that HSP90B1, the other form of HSP90, is involved in B cell signaling and its relation with the expression level of IgM has been established. This detected association provides genetic evidence for the regulatory role of self-antigens on the production of IgG NAbs at older ages, although the association with IgM NAbs was not observed.

Except for *IL6* and *IL13* exclusively associated with IgM and IgG, respectively, some other interleukins members were found associated with both isotypes. On GGA26, a potential QTL (SNPs rs14298901 and rs14298911) for IgG and IgM at older age (40 and 65 weeks of age) was found. SNPs rs14298901 and rs14298900 are located in *IL10* gene. IL10 is an anti-inflammatory cytokine produced primarily by monocytes, T-cells and B-cells (Moore et al., 1993), and influences multiple immunological mechanisms. Studies in murine models showed that, in general, low levels of IL10 increase resistance and high levels increase susceptibility to intracellular pathogens (Moore et al., 2001). In broilers, SNPs in *IL10* were strongly associated with Salmonella burden (Ghebremicael et al., 2008). SNP rs14298911 was in the sequence of *IL19*, which is tightly linked to IL10, and showed association with IgG40 and IgG65. IL19 is another cytokine that belongs to the IL10 family of cytokines (Yilmaz et al., 2005; Kim et al., 2009). The function of IL19 seems to be conserved in chickens as in mammals. IL19 plays a similar role as IL10 in response to intracellular poultry pathogens (Kim et al., 2009). The association of *IL10* and *IL19* with IgG and IgM at later age provides further evidence for the role of the IL10 family cytokines in immune response and survival at later ages.

3.4.5 Detected SNPs associated with other immune and behavior traits

Few of the SNPs or QTL for NAbs isotypes IgM and IgG detected in this study were also found to be associated with total NAbs titers (Biscarini et al., 2010a). The genetic correlations between total NAbs and individual isotypes are generally slightly positive: this can partly explain the few genetic polymorphisms found to be associated with both total NAbs titres and isotype titers and suggest total NAb and NAb isotype titers may be different traits. The total NAb (in serum) titer is a more comprehensive indicator for humoral innate immunity: IgM and IgG (and less IgA) isotypes are NAb sub-components, which offer a more focused and detailed view. In addition, total NAb titers were described to increase with age (Star et al., 2007), while the isotype titers seem to decrease with age (Table 3.1). Haghighi et al. (2006) found that the increase of NAb isotypes was not associated with an increase in total NAb in the intestines of probiotic-treated female broilers. This may further imply that as genetically different traits (current paper), total NAb and NAb isotype titers convey information about the different status of the animals' immune system.

Some SNPs or QTL found associated with IgM and IgG titers in this study were also reported to be significantly associated with other immune traits like SpAb response

and complement activity, specially the classical complement activation cascade (Biscarini et al., 2010b). NAbs and complement system are two interrelated humoral components of the innate immune defense. On one hand, the levels of complement and the expression of its receptors by B cells are positively correlated with NAb diversity and B-1 cell number (Carroll and Prodeus 1998). On the other hand, NAbs can activate the classical complement pathway by binding to invading microorganisms (Ochsenbein and Zinkernagel 2000; Austen et al., 2003; Jayasekera et al., 2007). In our study, a common genetic background of NAb isotype titers and complement activity was observed. Laying hens which were selected for high specific immune responsiveness also have a higher level of NAbs (Parmentier et al., 2002; Parmentier et al., 2004). A QTL on GGA13 (SNPs rs14064896 and rs15709619) affecting IgM and IgG NAbs was previously found to be associated with SpAb response to Newcastle disease in laying hens (Biscarini et al., 2010a), although the functional relationship between NAb isotypes and SpAb remains unclear. Our results, together with previous studies, indicated genetic relationships between multiple immune traits, thus contributing additional evidence to the interaction and cooperation of different immune components in health and disease. The likely different roles of NAb isotypes in the regulation of immune responses need however further investigation.

Biscarini et al. (2010b) found that some genetic regions associated with immunity - for instance with IL4 and IL9- also had an effect on behavioral traits. Some of the SNPs or QTL for IgM and IgG titers detected in this study were reported to be associated with feather pecking behavior by Biscarini et al. (2010b). In particular, they were all reported to be associated with the associative genetic effects (genetic effects of cage mates on the individual phenotype) on plumage condition score (an indirect measure of the pecking behavior), and none with the direct genetic effects. Examples include rs13512983 and rs16422070 on GGA4, rs13596168 on GGA7, and rs16105159 on GGAZ. This suggests that NAb isotype titers may be linked with the propensity to express pecking, but not with the susceptibility to receive it (see Biscarini et al., 2010b, for details). It is worthwhile to mention that most of these SNPs were associated with IgG20 and plumage condition scores at 51 weeks of age. SNPs associated with a natural immune trait and plumage condition score reveal possible relationships between the immune system and social behavior, as described previously (Parmentier et al., 2009; van der Poel et al., 2011).

3.5 Conclusions

In this study, moderate heritabilities were estimated for two different NAbs isotypes: IgM and IgG. Results indicated that selection for isotypes of NAbs is feasible: this could be especially relevant in the case of IgM20 and IgG20, which is related to survival through the laying period. An association study with 1,022 SNPs was performed across lines and revealed 43 significant associations for NAbs isotype titers at different ages. The lower extent of common linkage disequilibrium (LD) blocks conserved across lines increases the resolution of the association study, implying that detected markers are bound to be closer to QTL. Some SNPs were located in immune-related candidate genes like *IL10*, *IL19*, *HSP90AB1* and *IRF1*. To the best of our knowledge, this is the first time they are indicated to be associated with NAb isotype titers, regardless of species. Follow-up studies are needed to verify if they are causal factors for these traits in the current study. The observation that some detected SNPs were also associated with adaptive immune traits and behavioural traits, reveals a genetic basis for relationships between the innate and adaptive components of the immune system, and behaviour. In summary, our findings will provide preliminary information on the identifying of genes or related markers underlying of NAb isotype titers, which can be useful in genetic selection for higher survival in laying hens.

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4

Genetic parameters of natural antibody isotypes and survival analysis in beak trimmed and non-beak trimmed crossbred laying hens

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Abstract

Natural antibodies (NAb) are important humoral components of innate immunity. As first line of defense, NAb provide protection to infection and support adaptive immunity. An earlier study indicated that serum levels of NAb isotypes IgM and IgG at young age were predictive for survival in non-beak trimmed purebred laying hens during the laying period. In the present study, genetic parameters of NAb isotypes were estimated and relationships between survival and NAb isotypes levels in crossbred laying hens were investigated. In total, 1,555 beak trimmed and 1,169 non-beak trimmed crossbred laying hens were used. Genetic parameters of IgM and IgG titers binding keyhole limpet hemocyanin (KLH) at 24 weeks of age were estimated with a linear animal model. The heritabilities of NAb isotypes IgG and IgM were 0.21 (SE = 0.04) and 0.26 (SE = 0.04), respectively. The genetic correlation between IgG and IgM isotypes was 0.43 (SE = 0.11). These results indicated that NAb isotype titers were heritable traits in the crossbred laying hens. Both NAb isotypes can be selected for simultaneously as the detected positive genetic correlation (0.43, SE = 0.11) between them is positive. Both row and level of the cage were indicated to be associated environmental factors for NAb isotype titers. Different from an earlier study with purebred hens, survival analysis showed no significant associations of survival with NAb isotype titers in beak trimmed or non-beak trimmed crossbred hens. Non-health-related causes of mortality, especially in birds with intact beaks, overruled the anticipated relationships between NAb isotype titers and survival.

Key words: IgM, IgG, heritability, beak treatment, mortality

4.1 Introduction

Antibodies are defined as immunoglobulins produced by plasma cells after naïve B lymphocytes recognize antigen during infection or immunization with antigens including vaccines. Therefore, conventionally, antibodies are antigen-triggered and characterized by their antigen-specificity. In contrast, natural antibodies (NAb) are defined as antibodies being present in healthy individuals without any previous antigen exposure (Avrameas, 1991; Haury et al., 1997).

NAb are found in every species tested so far, like humans (Guilbert et al., 1982), mice (Ochsenbein et al., 1999), fish (Sinyakov et al., 2002), and chicken (Neu et al., 1984). This conservation during evolution suggests that NAb are not simple non-specific by-products of exogenous immunization but may play a vital physiological role (Cohen, 2007). Alike specific antibodies (SpAb), NAb can be either of the IgM, IgG (IgY), and IgA isotypes in birds. The broad reactivity of IgM, the principal NAb isotype, provides pre-existing defense which enables animals to rapidly recognize and protect against infection by pathogens that have not been encountered previously (Baumgarth et al., 2000). This protection fills the gap between the onset of infection and the emergence of the adaptive immune response (Baumgarth et al., 2000). For example, the antigen-induced antibodies to influenza virus can be detected in the serum at 5 days after infection (Baumgarth, 2000), whereas the pre-existing NAb can prevent major viral replication and consequent virus-induced tissue destruction (Ochsenbein et al., 1999). NAb facilitate specific immunity by activating the classical complement pathway (Ochsenbein et al., 1999), and capture and present antigen to T helper cells (Elluru et al., 2008). It was proposed that screening for NAb against pathogens may predict the strength of an antigen-induced immune response and could be used as a tool for vaccine development (Kohler et al., 2003). NAb were reported to react with self or foreign novel molecules (Quintana and Cohen, 2004). Keyhole limpet hemocyanin (KLH) is large metalloprotein and used as an example of a naïve antigen for detecting NAb in laying hens (Parmentier et al., 2004; Star et al., 2007).

In an earlier study with non-beak trimmed laying hens from multiple purebred White Leghorn lines and Rhode Island Red lines, serum levels of NAb, especially IgM binding KLH at 20 weeks of age, were found to be significantly associated with and predictive for survival during the laying period, i.e. the higher the titers of NAb at young age, the higher the probability of layers to survive (Sun et al., 2011). The odds ratios estimated for the isotypes IgM and IgG as factors in the survival analysis also indicated that distinguishing isotype titers is less predictive for the survival in

White Leghorns than in the Rhode Island Red layers. A hypothesis for different prediction power of NAb isotype titers in the two breeds was that NAb isotype titers are associated with the health-related survival but in non-beak trimmed White Leghorns, part of death may be caused by non-health-related reasons, such as severe feather pecking and cannibalism. Therefore, in the present study, we investigated the relationships between NAb isotype titers binding KLH and survival in beak trimmed and non-beak trimmed crossbred laying hens. Genetic parameters for the NAb isotype titers were also estimated. Crossbred laying hens are more common as commercial product than purebred ones in the poultry industry. Identifying the predictive parameters for survival and better understanding of the genetic parameters in crossbred hens will be valuable for designing a breeding strategy for improved survival.

4.2 Materials and methods

4.2.1 Study population

Female crossbred offspring of two commercial purebred White Leghorn layer lines (W1 and WB) with pedigree information was provided by the Institut de Sélection Animale (ISA) B.V., the layer breeding division of Hendrix Genetics (Boxmeer, The Netherlands). Fifty sires of line W1 were randomly chosen and mated with 908 dams of line WB. Dams and sires were housed individually. Each sire was mated to approximately 18 dams, and each dam contributed on average 3 female offspring, resulting in 2,859 offspring.

4.2.2 Housing and management

All chickens from the cross between W1 and WB lines were hatched, sexed and wing-banded, respectively, at the same time. Only female chicks were kept for this study. The offspring of 25 sires were beak trimmed whereas the offspring of another 25 sires were kept with intact beaks. Chicks were trimmed manually at day old using a hot blade to remove and cauterize the tip of the beak. Hens were allocated to rearing cages randomly with respect to beak trimming, 60 individuals per cage. From 5 weeks of age onwards, the hens were housed with 20 individuals per cage. At 17 weeks of age, all hens were transported to a high-light intensity laying house with battery cages. There were three double rows of cages in the laying house, with rows in between to allow employees to have access to the cage. The outer two double rows consisted of three levels (top; middle, closest to the light; and bottom). The inner double rows consisted of four levels (super top; top;

middle, closest to the light; and bottom). Hens were only placed in the top and middle levels (Figure 4.1). Five half-sibs or full-sibs with the same beak treatment were allocated to a single cage. Water and standard commercial layer diet was provided *ad libitum*. The chickens started with a 9L : 15D light scheme, and increased 1 hour per week until 16L : 8D was reached when the hens were 26 weeks of age. The hens received routine vaccinations for Marek's disease (day 1), infectious bronchitis (day 1, week 2, 10, 12 and 15), Newcastle disease (week 2, 6, 12 and 15), infectious bursal disease (week 3 and 15), turkey rhinotracheitis (week 8 and 18), fowl pox (week 15), chicken anaemia virus (week 15) and avian encephalomyelitis (week 15).

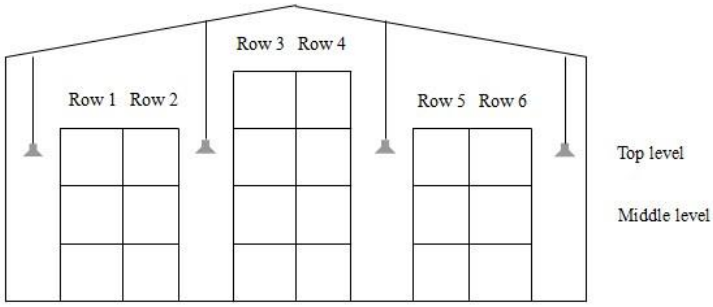

Figure 4.1 The division of the stable.

4.2.3 Study design

All hens were observed daily from 17 until 83 weeks of age. Hens that died were removed from the cages and not replaced. Wing-band number, cage number, and date of death were recorded. Cause of death was not determined. For each hen, information was collected on survival and number of survival days. Survival was defined as dead (0) or alive (1) at the end of the study. From these data, survival rate was calculated as the percentage of laying hens still alive at the end of the study. Survival days were defined as the number of days from the start of the observation until either death or termination of the study, with a maximum of 457 days (actual age of 581 days, since the date of hatch).

4.2.4 NAb isotypes IgM and IgG titers binding KLH

At 24 weeks of age, blood samples from all hens were taken from the wing vein for measurements of titers of NAb isotypes IgM and IgG binding KLH in the serum using ELISA as described earlier (Sun et al., 2011).

4.2.5 Data analysis

Descriptive Statistics. Descriptive statistical analyses were performed using SAS 9.1.2 (SAS Institute, 2004). Effects were considered significant at $P < 0.05$. A multiple comparison test (Bonferroni) was conducted to study the differences in NAb isotype IgM and IgG titers binding KLH in contrast groups.

Genetic Parameters Estimation of NAb Isotype Titers Binding KLH. A linear animal model was used to estimate variance components, heritability of NAb isotype IgM and IgG for the whole population including both beak trimmed and non-beak trimmed laying hens, using the ASReml program (Gilmour et al., 2006). Fixed effects were established using GLM with SAS 9.1.2 (SAS Institute, 2004). Fixed effects considered in this study were beak treatment, row and level of the cage. The final model for the variance components estimation was:

$$y_{ijk} = \mu + row_i + level_j + a_k + e_{ijk} \quad (1),$$

where y_{ijk} was NAb isotype IgM or IgG titers binding KLH; μ was the overall mean, row_i was the fixed effect of row of the cage (i = 1, 2, 3). There were six rows as shown in Figure 4.1. Row 1 and 6, row 2 and 5, and row 3 and 4 were treated as the same row, respectively); $level_j$ was the fixed effect of level of the cage (j = 1, 2); a_k was the random additive genetic effect of the animal k (direct genetic effect); and e_{ijk} was the residual term. (Co)variance structures of model terms are $\mathrm{var}(\boldsymbol{a}) = \boldsymbol{A}\sigma_a^2$, with *A* being the additive genetic relationship matrix based on four generations of ancestors extracted from the pedigree file provided by ISA, and σ_a^2 is the additive genetic variance and $\mathrm{var}(\boldsymbol{e}) = \boldsymbol{I}\sigma_e^2$, in which ***I*** is an identity matrix, σ_e^2 is the residual variance. Genetic and phenotypic correlations between traits were estimated based on bivariate analyses using equation (1). The heritability for NAb isotype titers was calculated as $h^2 = \sigma_a^2 / (\sigma_a^2 + \sigma_e^2)$, and phenotypic variance was $\sigma_P^2 = \sigma_a^2 + \sigma_e^2$.

Survival Analysis with NAb Isotype Titers as Explanatory Variables. Multivariable multilevel logistic regression analyses were used to assess the relationships between survival (binary variable taking the values 0 for survived and 1 for dead) from 24 to 83 weeks of age and NAb isotypes IgM or IgG titers binding KLH at 24 weeks of age in beak trimmed hens and non-beak trimmed laying hens, respectively. The original logistic regression model for both populations was:

$$\log it(\pi) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \varepsilon \quad (2),$$

where $\log it(\pi) = \ln(\pi / (1-\pi))$, π = probability to die between 24 and 83 weeks of age given a set of explanatory variables: x_1 = effect of IgM titers binding KLH at 24 weeks of age, x_2 = effect of IgG titers binding KLH at 24 weeks of age, x_3 = effect of row of cage, x_4 = effect of level of cage. β_0 is the intercept from the equation (the value of the criterion when the explanatory variables are equal to zero), β_1, …, β_4 are the regression coefficients indicating the relative effect of a corresponding explanatory variable (x_1, …, x_4) on the outcome. The continuous variables IgM, IgG titers were inspected for linearity in the log-odds by dividing them into classes. The Likelihood Ratio Test was used for the significance of variables. The other covariates included row and level of the cage where the laying hens were located. Non-significant covariates ($P > 0.05$) were removed from the model one by one starting with the effect showing the highest P-value. If a removed covariate was deemed a confounder (i.e. one or more regression coefficients of the remaining variables relatively changed over 25%) it was forced back into the model. The fit of the logistic models was assessed by the Hosmer and Lemeshow Goodness-of-Fit Test (Hosmer and Lemeshow, 1989). Outcomes of logistic regression analyses were presented as odds ratios, which indicate the ratio of risks to die dependent on the titers of IgM and IgG binding KLH (Sun et al., 2011).

4.3 Results

4.3.1 NAb isotype titers in beak trimmed and non-beak trimmed laying hens

Before the serum samples were collected at 24 weeks of age, 135 laying hens died. In total, NAb isotype IgM and IgG titers were determined in 1,555 beak trimmed and 1,169 non-beak trimmed hens (Table 4.1). The NAb IgG titers in non-beak trimmed hens were significant higher than that in beak trimmed ones. There was no significant difference for IgM titers between two populations. There were no significant differences for IgM titers between the surviving and non-surviving hens within either population. However, in beak trimmed hens, IgG titers in non-surviving was significant higher than that in the surviving hens (Table 4.1).

Average IgG and IgM isotype titers of both beak trimmed and non-beak trimmed hens located at top level were significantly higher than the hens located at middle level. A significant difference of the average IgG and IgM isotype titers of both beak

trimmed and non-beak trimmed hens was also observed among the row categories where the cages were located: the hens located in the middle two rows (row category 3) of the house had higher NAb isotype titers (Table 4.1).

Table 4.1 Number (n), average natural antibody (NAb) isotype IgM and IgG titers binding keyhole limpet hemocyanin (KLH) with SD[1] in beak trimmed and non-beak trimmed laying hens.

Population	Variable	Class	n	IgM titers	IgG titers
Non-beak trimmed	Survival	Survival	813	8.17 (1.06)	6.69 (1.16)
		Non survival	356	8.18 (1.10)	6.77 (1.24)
	Level	Middle	578	8.02 (1.01) ^{b}	6.47 (1.13) ^{b}
		Top	591	8.33 (1.11) ^{a}	6.95 (1.18) ^{a}
	Row category	1	398	8.13 (1.14) ^{b}	6.55 (1.12) ^{b}
		2	398	8.03 (1.10) ^{b}	6.42 (1.19) ^{b}
		3	373	8.38 (0.94) a	7.20 (1.09) ^{a}
	Total		1,169	8.17 (1.07)	6.71 (1.18) ^{x}
Beak trimmed	Survival	Survival	1,447	8.24 (1.05)	6.59 (1.22) ^{b}
		Non survival	108	8.30 (1.03)	6.86 (1.16) ^{a}
	Level	Middle	802	8.13 (0.95) ^{b}	6.44 (1.22) ^{b}
		Top	753	8.36 (1.13) ^{a}	6.79 (1.19) ^{a}
	Row category	1	525	8.18 (1.04) ^{b}	6.51 (1.16) ^{b}
		2	513	8.11 (1.11) ^{b}	6.39 (1.22) ^{b}
		3	517	8.42 (0.95) ^{a}	6.93 (1.22) ^{a}
	Total		1,555	8.24 (1.05)	6.61 (1.22) ^{y}

$^{a\ b}$ Different superscripts indicate there is significant difference between different classes within the same variable ($P < 0.05$).

$^{x\ y}$ indicate there is significant difference for all beak trimmed and non-beak trimmed laying hens ($P < 0.05$).

[1] SD are in parentheses.

4.3.2 Survival of beak trimmed and non-beak trimmed crossbred laying hens

Table 4.2 shows the survival and average survival days of beak trimmed and non-beak trimmed hens. The beak trimmed hens had high survival rate of 93.1% and average survival days of 447.8 days. The hens with intact beaks had low survival

rate of 69.5% and average survival days of 383 days. The difference for survival and survival days in the two populations was significant. Kaplan-Meier survival function ($S(t) = P(T \geq \tau)$) curves also show significant different survival experience versus time for beak trimmed and non-beak trimmed laying hens (Figure 4.2). In Figure 4.3, the probability density function curve for non-beak trimmed laying hens increased sharply from 18 to 26 weeks of age, and from 34 to 36 weeks of age (at the peak of egg production). This indicates that the death rate increased during these time periods. The increase from 18 to 26 weeks of age might be because the laying hens were moved to new cages, meeting unfamiliar cage mates and experiencing increased light intensity. The increase from 34 to 36 weeks of age might be caused by the increasing production of eggs. In beak trimmed laying hens, the probability of death was highest at 63 weeks of age, which might be caused by aging.

Table 4.2 Number (n), survival and average survival days (day) with SD[1] for beak trimmed and non-beak trimmed crossbred laying hens.

Population	Animals	n	Survival (%)[2]***	Survival days (day)[3]***
Non-beak trimmed	Total	1,169	69.5 (1.4)	383.0 (4.0)
	Survival	813	--	457.0 (0.0)
	Non survival	356	--	214.2 (7.3)
Beak trimmed	Total	1,555	93.1 (0.6)	447.8 (1.2)
	Survival	1,447	--	457.0 (0.0)
	Non survival	108	--	324.3 (11.6)

*** $P < 0.0001$

[1] SD are in parentheses.

[2] Survival is the percentage of hens still alive at the end of the study (83 weeks of age).

[3] Survival days are the average number of days from day of observation (17 weeks of age) till death or the end of the study, with a maximum of 457 days.

4.3.3 Genetic parameters estimation for NAb isotype titers binding KLH

In beak trimmed and non-beak trimmed laying hens, heritability of NAb isotypes IgG and IgM titers binding was 0.21 (SE = 0.04) and 0.26 (SE = 0.04), respectively (Table 4.3). Genetic and phenotypic correlations between IgG and IgM titers were estimated to be 0.43 (SE = 0.11) and 0.28 (SE = 0.02), respectively, based on bivariate analyses using equation (1).

4.3.4 Relationship between NAb isotype titers and survival in beak trimmed and non-beak trimmed laying hens

The final variables for survival analysis in beak trimmed and non-beak trimmed laying hens are shown in Table 4.4. In beak trimmed hens, row of the cage was neither a significant factor (P = 0.29) nor an important confounder and thus was removed from the logistic model. The fit of the ordinary logistic model was sufficient (Chi-square = 2.86, P = 0.90). Level was a significant factor (P = 0.002). Preliminary analysis indicated that IgM and IgG titers at 24 weeks of age were not linearly related to the survival of laying period and were therefore categorized into 5 classes, with IgG titers < 5.0 and IgM titers < 7.0 as reference class, respectively. The odds ratios for higher IgM or IgG titers groups (except for the IgM titers group 8.0-8.9) were smaller than 1.00, but not significant (P > 0.05) (Table 4.4).

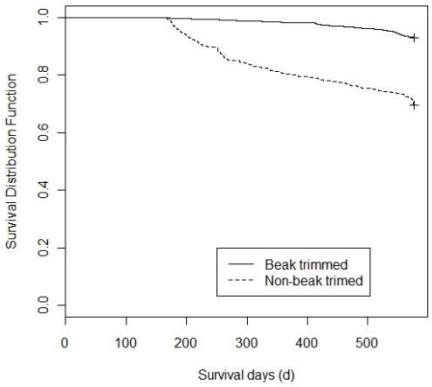

Figure 4.2 Kaplan-Meier survival function ($S(t) = P(T \geq \tau)$) curves for beak trimmed and non-beak trimmed laying hens. Vertical axis represents estimated probability of survival. Horizontal axis is the actual survival days of the laying hens. Any point on the curve gives the percentage surviving at a particular time. The Log-rank test and Wilcoxon test were used to compare survival curves which both indicate significant difference of survival experience of beak trimmed and non-beak trimmed laying hens.

In non-beak trimmed laying hens, row and level of the cage had no direct significant effect on survival (P = 0.08 for both variables). However, when removing them from the model, the relative change of the coefficient for IgM and IgG titers at 24 weeks of age was larger than 25%. So row and level of the cage were both included in the final model to get proper estimation for NAb isotype titers. The fit of the ordinary logistic model was not sufficient (Chi-square = 6.31, P = 0.61). The IgM and IgG titers at 24 weeks of age were not linearly related to the survival of laying period and were therefore categorized in 5 classes, with IgG titers < 5.0 and

IgM titers < 7.0 as reference class, respectively. The odds ratios for higher IgM titers groups (except for the group 9.0-9.9) were smaller than 1.00, but not significant ($P > 0.05$). The odds ratios for higher IgG titers groups were larger than 1.00, but not significant either ($P > 0.05$) (Table 4.4).

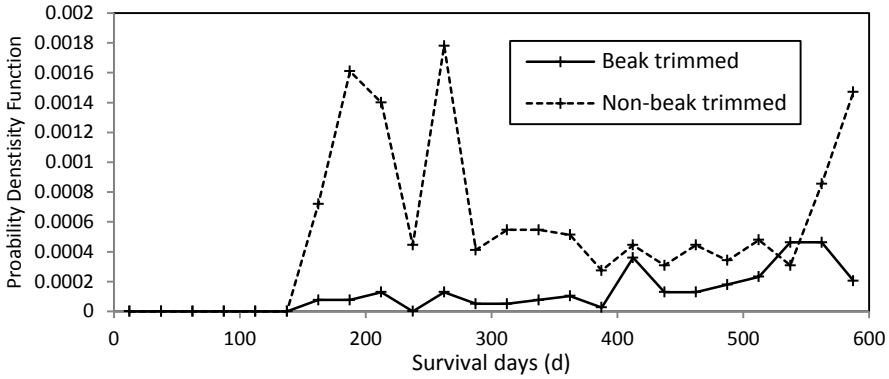

Figure 4.3 Probability density function curves of beak trimmed and non-beak trimmed laying hens. The probability density function is the first derivative of the cumulative probability function (f(t) = dF(t) / d(t) = -dS(t) / d(t). For a given interval the surface underneath the curve gives the probability that the time it takes to die within that interval. Alternatively, it can be thought of as the instantaneous probability of dying at specific time.

Table 4.3 Estimates of genetic parameters1 with SE2 for natural antibody (NAb) isotype IgM and IgG titers binding keyhole limpet hemocyanin (KLH) at 24 weeks of age in crossbred laying hens.

	IgG titers	IgM titers
σ_a^2	0.29 (0.06)	0.28 (0.05)
σ_e^2	1.06 (0.05)	0.81 (0.04)
σ_P^2	1.35 (0.04)	1.10 (0.03)
h^2	0.21 (0.04)	0.26 (0.04)
r_g	0.43 (0.11)	
r_p	0.28 (0.02)	

[1] σ_a^2 is the additive genetic variance, σ_e^2 is the random residual variance, σ_P^2 is the phenotypic variance: $\sigma_p^2 = \sigma_a^2 + \sigma_e^2$, h^2 is the heritability: $h^2 = \sigma_a^2 / (\sigma_a^2 + \sigma_e^2)$, r_g is the genetic correlation and r_p is the phenotypic correlation.
[2] SE are in parentheses.

Table 4.4 Multivariable multilevel logistic regression analysis of IgM and IgG titers in serum binding keyhole limpet hemocyanin (KLH) at 24 weeks of age, for survival of 24 to 83 weeks of age in beak trimmed and non-beak trimmed laying hens.

Population	Variable	Class	n	Odds Ratio	95% Confidence Interval	*P*-value
Beak trimmed	IgM titers	< 7.0	174	1.00	Ref [1]	Ref
		7.0 – 7.9	399	0.69	0.33-1.44	0.33
		8.0 – 8.9	583	1.29	0.60-2.76	0.51
		9.0 – 9.9	337	0.79	0.37-1.70	0.54
		> 10.0	62	0.86	0.27-2.69	0.79
	IgG titers	< 5.0	91	1.00	Ref	0.45
		5.0 – 5.9	377	0.62	0.18-2.15	0.23
		6.0 – 6.9	441	0.47	0.14-1.59	0.11
		7.0 – 7.9	443	0.37	0.11-1.26	0.27
		> 8.0	203	0.48	0.13-1.76	0.45
	Level	Middle	802	1.00	Ref	Ref
		Top	753	0.50	0.31-0.77	0.002
Non-beak trimmed	IgM titers	< 7.0	142	1.00	Ref	Ref
		7.0 – 7.9	329	0.76	0.49-1.19	0.24
		8.0 – 8.9	426	0.82	0.53-1.27	0.37
		9.0 – 9.9	228	1.21	0.73-1.99	0.46
		> 10.0	44	0.80	0.38-1.68	0.55
	IgG titers	< 5.0	52	1.00	Ref	Ref
		5.0 – 5.9	274	1.47	0.77-2.81	0.24
		6.0 – 6.9	323	1.15	0.60-2.18	0.68
		7.0 – 7.9	347	1.04	0.55-1.99	0.90
		> 8.0	173	1.07	0.54-2.15	0.84
	Row category	1	398	1.00	Ref	Ref
		2	398	0.74	0.54-1.01	0.06
		3	373	0.72	0.52-1.00	0.05
	Level	Middle	578	1.00	Ref	Ref
		Top	591	0.80	0.61-1.03	0.08

[1] Ref indicated a reference class.

4.4 Discussion

4.4.1 Genetic analysis of NAb isotype titers binding KLH in crossbred laying hens

In crossbred laying hens, moderate heritabilities were found for NAb isotypes: 0.21 (SE = 0.04) for IgG and 0.26 (SE = 0.04) for IgM. This indicated that NAb isotype titers in crossbred birds show genetic variation. In a previous study, the heritability for IgG and IgM titers binding KLH at 20 weeks of age was estimated as 0.31 and 0.41, respectively in purebred laying hens (Sun et al., 2013). The difference may rest on different populations, and slight differences of the traits studied (isotype titers at 24 week in the present and 20 weeks in the previous study). The heritability for IgM was higher than IgG in our study. This is in accordance with our previous study, and the possible explanations were discussed by Sun et al. (2013). The positive genetic and phenotypic correlation between IgM and IgG isotype was also observed in the previous study with purebred birds (Sun et al., 2013). The moderate genetic correlation suggests that IgM and IgG share partially the same genetic background but are relatively independently controlled. However, the positive genetic correlations indicated that two isotypes may be selected for simultaneously. Selection for improved NAb isotype titers is possible by selection in the purebred lines based on NAb measured in crossbreds, if the genetic correlation between the traits in purebred and crossbred lines is high. Our dataset only contained NAb isotype titers measured on crossbred offspring, it was, therefore, not possible to estimate this genetic correlation. It is, however, important to estimate the genetic correlation before developing a strategy to breed for enhanced NAb titers in crossbred birds.

Direct heritability can be overestimated when maternal effects exist but are neglected in model analysis (Clement et al., 2001; Kruuk and Hadfield, 2007). Transfer of maternal immune functions across generations was suggested (Grindstaff et al., 2003; Staszewski and Siitari, 2010). In poultry, 10% of the NAb levels variation was attributed to maternal environmental effects by Wijga et al (2009). In the present study, the limited number (2 to 5) of offspring per dam made it difficult to detect a maternal effect. However, heritabilities estimated for NAb isotype titers from the dam model (with dam effect being the only random effect in the model besides the residual, assuming dam unrelated) were higher than those from traditional animal models (data not shown). This indicated that a fraction of the variation could be attributed to maternal environmental effects.

Row and level of the laying hens cage were established as fixed effect for NAb isotype titers to avoid overestimated heritabilities for the traits. Laying hens located in top level had significant higher isotype titers than those in middle level (Table 4.1). Laying hens located in two middle rows (row category 3) had significant higher isotype titers than those in the four side rows (row category 1 and 2) (Table 4.1). The laying hens located in different levels and row categories may receive different light intensity which affected the production of a hormone like melatonin. The bilateral interactions between the hormone and immune system were reported before (Guerrero and Reiter, 1992; Skwarlo-Sonta, 1996). Furthermore, the laying hens located in different row categories may receive different extent of employee intervention as employee had access to the cage through rows in between which could cause stress for the birds. Effects of stress on the immune system were reported before (Gross and Siegel, 1983; Poliak et al., 1997). The finding of these associated environmental factors (housing) for NAb isotype titers show that these factors should not be ignored in future studies (Gross and Siegel, 1983).

4.4.2 NAb isotype titers is not predictive for survival in beak trimmed or non-beak trimmed crossbred laying hens

Total NAb titers and isotype IgM and IgG titers binding KLH at 20 weeks of age were shown to be significantly associated with and predictive for survival in purebred hens in the laying period in previous studies (Star et al., 2007; Sun et al., 2011). In egg-production industry, commercial laying hens are usually crossbred. To investigate the predictive value of NAb isotype titers for survival in crossbred hens, a population of crossbred female offspring from purebred W1 (male) and WB (female) lines was used. W1 line was typed to be a “high” NAb line, whereas WB line was a “low” NAb line (Star et al., 2007; Sun et al., 2011). The survival of W1 line was higher than that of WB line (Ellen et al., 2008). These observations were in line with our previous study that high NAb isotype IgM and IgG titers were associated with higher survival of laying hens. In the present study, the survival of non-beak trimmed laying hens was significantly higher than that of beak trimmed laying hens (Table 4.2 and Figure 4.2, 4.3). There was no significant difference of IgM titers in the two populations, but the IgG titers in non-beak trimmed laying hens were significantly higher (Table 4.1). We performed multivariable multilevel logistic analysis of IgM and IgG titers at 24 weeks of age for survival of 24 to 83 weeks of age. In beak trimmed laying hens, odds ratios of smaller than 1.00 were found for all IgG and IgM isotype groups (except for the IgM titer group 8.9-9.9), which

indicated that survival was higher in animals with higher antibody titers, but the associations were not significant, and titers and survival were not completely linear related. In the non-beak trimmed laying hens, odds ratios of lower than 1.00 were found for IgM titer groups (except for the titer group 9.9-10.0), which indicated that the survival was lower for the hens with lowest IgM titers. Different from the finding in beak trimmed laying hens, odds ratios larger than 1.00 were found for all IgG titer groups. This indicated that survival was higher for animals with lower IgG titers, although all the associations were not significant and there was no completely linear relationship between survival and isotype titers. In the present study, we also estimated the breeding value (EBV) of the 50 sires for NAb isotype titers and investigated the linear regression between sire EBV and the survival of their offspring. Beak trimmed laying hens showed high survival irrespective of the sire EBV for NAb isotypes. Sires with higher EBV for NAb isotype titers did not always predispose their non-beak trimmed offspring for higher survival either. However, the regression indicated that survival of offspring was higher for the sires with higher EBV for IgM, whereas survival was lower for the sires with higher EBV for IgG (see Appendix). This was in agreement with the logistic regression analysis.

In general, the observations from logistic regression analysis were not consistent with our previous findings, where odds ratio of 0.56 ($P < 0.0001$) and 0.72 ($P = 0.02$) was found for IgM and IgG, respectively in Brown purebred laying hens and odds ratio of 0.74 ($P = 0.01$) and 0.99 ($P = 0.99$) was found for IgM and IgG, respectively in White Leghorn purebreds (Sun et al., 2011). As we hypothesized previously, different causes of mortality in two populations may account for this. In non-beak trimmed laying hens, death rate increased from 18 to 26 weeks of age, and from 34 to 36 weeks of age. The increase from 18 to 26 weeks of age might be because the laying hens were moved to new cages, meeting unfamiliar cage mates and experiencing increased light intensity. The increase from 34 to 36 weeks of age might be caused by the increasing production of eggs. These reasons were causing factors of feather pecking. Therefore, mortalities in non-beak trimmed laying hens are likely mainly due to cannibalism, which is considered the ultimate phase of severe feather pecking. In the study of Peeters et al. (2012), as high as 99.7% (6,683 out of 6,706 crossbred laying hens with intact beak) of dead animals was related with feather pecking behaviour or cannibalism. Beak trimming is an effective way of preventing the severe feather pecking and thus improve the survival of laying hens (Table 4.2 and Figure 4.2). Based on our observation, the feather condition of beak trimmed laying hens was also significantly better than those in non-beak trimmed ones (data not shown). Analysis of survival days for the two populations in

the present study using direct-associative effect animal models (Bijma et al., 2007) indicated that in non-beak trimmed laying hens, the heritable variation for survival days was 6-fold larger than that in beak trimmed laying hens, and almost all variation was contributed by the associative effect (data not shown). Therefore, while some of the deaths of non-beak trimmed laying hens may be due to health-related causes, the majority of death in this population was caused by harmful social interactions, such as feather pecking and cannibalism. In the earlier study, NAb isotype titers were also shown to be more sensitive and acute parameters for survival in the Brown laying hens, which show much less feather pecking behavior than White Leghorn laying hens (Uitdehaag et al., 2008). These results indicate more complex relationships between NAb isotype titers and survival of the layers population when the cause of mortality is also complex.

In beak trimmed crossbred hens in the present study, mortality was less likely caused by severe feather pecking. The survival was significantly higher than that in non-beak trimmed hens (Table 4.2 and Figure 4.2). However, the association between the NAb isotype titers and survival was not significant either (Table 4.4). This different observation from that in purebred laying hens, may rest on crossing. In the present study, a survival of 93.1% was found for beak trimmed crossbred offspring. In the study of Star et al. (2007), a survival of 89.6% and 87.1% was found for paternal W1 and maternal WB line, respectively. The higher survival of crossbred offspring indicated a heterosis for survival. It is possible that heterosis has a more prominent effect on survival than the NAb isotypes levels in the offspring.

4.5 Conclusions

NAb isotypes IgM and IgG titers binding KLH at 24 weeks of age are heritable traits in crossbred laying hens. Maternal environmental effects on isotype titers are also indicated. Environmental factors including row and level of the cage (light intensity) were associated with NAb isotype titers. Different from purebred laying hens, there was no significant association between NAb isotype titers and survival in beak trimmed or non-beak trimmed crossbred laying hens. The present results confirmed our previous hypothesis that non-health-related causes of mortality (severe feather pecking) overruled the anticipated relationships between NAb isotype titers and survival in birds with intact beaks.

4.6 Acknowledgements

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4.7 Appendix

The breeding value (EBV) of the 50 sires for IgM and IgG titers, respectively was estimated form the following sire model implemented in the ASReml software package (Gilmour et al., 2006): $\boldsymbol{y} = \boldsymbol{Xb} + \boldsymbol{Zs} + \boldsymbol{e}$, where ***y*** is a vector of IgM or IgG titers, ***b*** is a vector of fixed effects, with incidence matrix ***X*** linking IgM or IgG titers to fixed effects; ***s*** is a vector of the sire effect (half of breeding values), with incidence matrix ***Z*** linking IgM or IgG titers to the sire effect; $\mathrm{var}(s) = A_s\sigma_s^2$, with ***A*** being the sire additive genetic relationship matrix, σ_s^2 being the sire genetic variance; $\mathrm{var}(e) = I\sigma_e^2$, with ***I*** being an identity matrix, σ_e^2 being the residual variance.

The survival of the offspring of the 50 sires was linearly regressed on the EBV of the sires for IgM (Figure 4.4A) and IgG (Figure 4.4B) titers. The linear regression models were also shown on the trend lines.

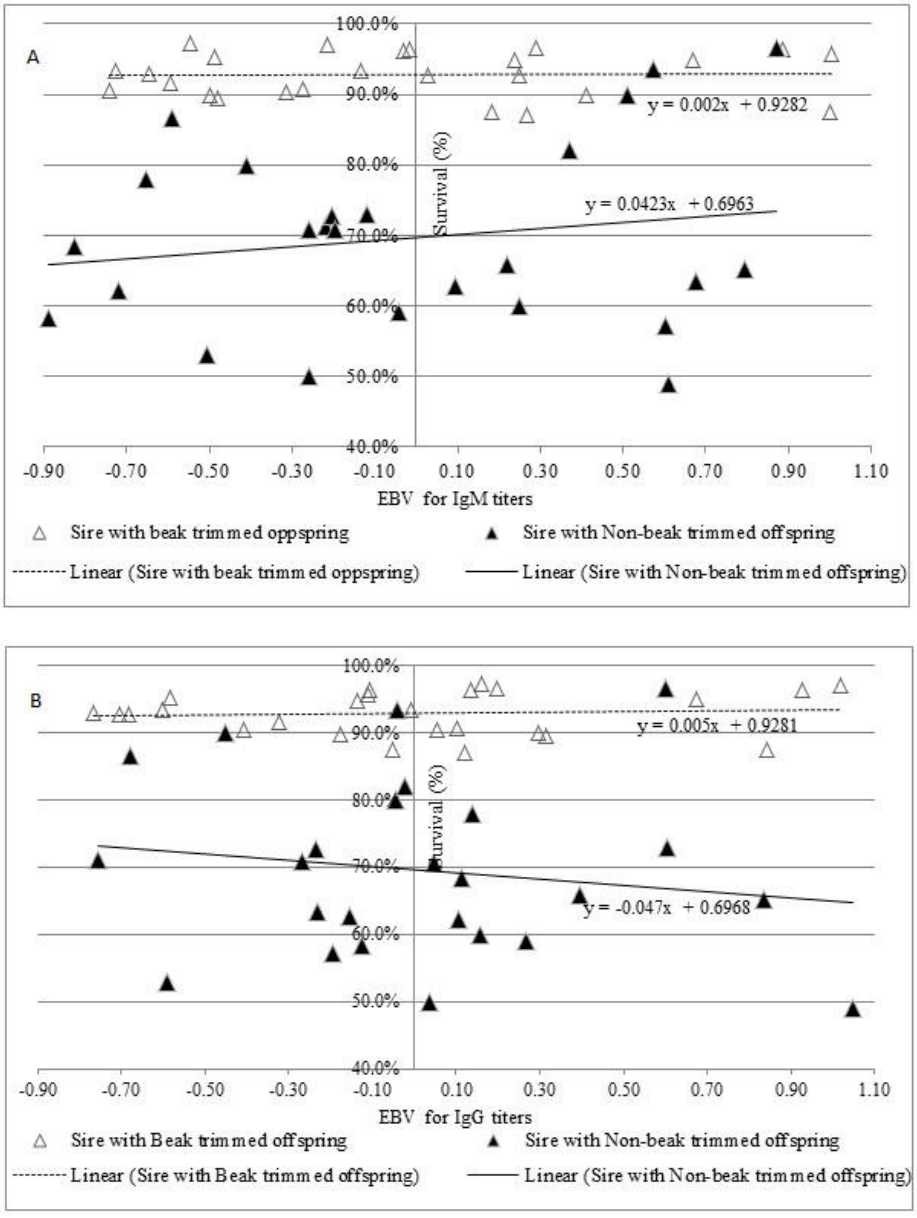

Figure 4.4 The regression of survival of the offspring on the estimated breeding value (EBV) for NAb isotype IgM titers (Figure 4.4A) and IgG titers (Figure 4.4B) binding keyhole limpet hemocyanin (KLH). Linear regression model was shown on the trend line.

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5

Modelling of feather pecking behaviour in beak trimmed and non-beak trimmed crossbred laying hens: variance component and trait-based approach

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Abstract

Natural antibodies (NAb) are important humoral components of innate immunity. As first line of defense, NAb provide protection to infection and support adaptive immunity. An earlier study indicated that serum levels of NAb isotypes IgM and IgG at young age were predictive for survival in non-beak trimmed purebred laying hens during the laying period. In the present study, genetic parameters of NAb isotypes were estimated and relationships between survival and NAb isotypes levels in crossbred laying hens were investigated. In total, 1,555 beak trimmed and 1,169 non-beak trimmed crossbred laying hens were used. Genetic parameters of IgM and IgG titers binding keyhole limpet hemocyanin (KLH) at 24 weeks of age were estimated with a linear animal model. The heritabilities of NAb isotypes IgG and IgM were 0.21 (SE = 0.04) and 0.26 (SE = 0.04), respectively. The genetic correlation between IgG and IgM isotypes was 0.43 (SE = 0.11). These results indicated that NAb isotype titers were heritable traits in the crossbred laying hens. Both NAb isotypes can be selected for simultaneously as the detected positive genetic correlation (0.43, SE = 0.11) between them is positive. Both row and level of the cage were indicated to be associated environmental factors for NAb isotype titers. Different from an earlier study with purebred hens, survival analysis showed no significant associations of survival with NAb isotype titers in beak trimmed or non-beak trimmed crossbred hens. Non-health-related causes of mortality, especially in birds with intact beaks, overruled the anticipated relationships between NAb isotype titers and survival.

Key words: laying hen, beak trimming, feather pecking, feather condition score, NAb

5.1 Introduction

During the past decades, poultry breeders have successfully improved the production performance, either egg production in layers or meat production in broilers. However, considering the ever-increasing social concern, future animal husbandry is also required to pay more attention to enhancing animal welfare. In laying hens, animal welfare is particularly focused on feather pecking behaviour. Feather pecking is defined as pecking towards the plumage of other birds. Two major forms of feather pecking can be distinguished: gentle and severe feather pecking (Keeling 1995). Severe feather pecking causes damage to the birds, results in bald patches, denuded area, haemorrhage, wounds, is painful for the birds, and can even lead to death (Gentle and Hunter 1991). Feather pecking is not only a welfare but also a serious economic problem (Rodenburg et al., 2008). Decreased egg production caused by feather pecking was observed (Johnsen et al., 1998). Feather loss because of feather pecking can lead to heat loss, which results in higher maintenance energy requirements (Blokhuis and Wiepkema, 1998). Mortalities due to cannibalism, which is considered the ultimate phase of severe feather pecking, can be substantial. Hill (1986), for example, found up to 15% mortality in laying hens housed in aviaries, while Peeters et al. (2012) and Ellen et al. (2008) found around 32-48% mortality due to cannibalism in cage-housed birds. Prohibition of both cage housing system and beak trimming because of animal welfare concern in many European Union member countries increase the risk of feather pecking and cannibalism.

Better understanding of the genetic and biological mechanisms of feather pecking is needed to find alternative ways of preventing this unfavorable behaviour. Feather condition score (FCS) is a measure of feather damage, which has been shown to be closely related to feather pecking behavior in hens housed in groups (Bilcik and Keeling, 1999; Uitdehaag et al., 2008). Different from ordinary traits, FCS is a so-called interacting phenotype, a trait whose value is also affected by the behaviour of an individual's conspecifics (the cage mates which are kept with the focal individual in the same cage in case of laying hens) (Moore et al., 1997). In contrast to the direct genetic effect of an individual on its own phenotype, the heritable effect of an individual on the phenotype of a conspecific is known as associative effect or indirect genetic effect (Griffing, 1967; Wolf, 2003; Bijma et al., 2007). Associative effects influence a trait's inherence and contribute to heritable variation (Moore et al., 1997; Bijma, 2011). For the genetic parameters estimation of survival days in non-beak trimmed laying hens, the inclusion of associative effects in the model gave higher heritable variation than a traditional linear animal

model (Muir, 2005; Ellen et al., 2008; Peeters et al., 2012). Consequences of feather pecking behaviour in beak trimmed and non-beak trimmed laying hens are different, and therefore FCS can be a different trait in both types of birds. In the first part of the present study, we estimated genetic parameters for FCS in beak trimmed and non-beak trimmed laying hens, respectively, with two variance components models: a traditional linear model and a linear animal model combining direct and associative effects.

approach can help to understand the biological mechanism of social interactions (Moore et al.,1997; Wolf et al., 1998). Knowledge of the traits that underlie the interacting phenotype is, however, needed for the trait-based approach (Kirkpatrick and Lande, 1989). Brain serotonergic levels (Chaouloff, 2000) and some neurotransmitters like dopamine and hormones (Cheng et al., 2003) were correlated with feather pecking. El-Lethey et al. (2003) found that feather pecking was related with corticosterone levels, which also reduced immune responses. Recently, the effects of immunity on feather pecking behavior were suggested by several studies. Buitenhuis et al. (2004) reported a significant genetic and phenotypic correlation between feather pecking and primary antibody response to keyhole limpet hemocyanin (KLH). Parmentier et al. (2009) found that when chickens were challenged intratracheallly and repeatedly at a young age with different doses of endotoxin lipopolysaccharide (LPS) and the protein human serum albumin (HuSA), they showed different levels of feather damage at an older age. In addition, some SNPs or QTLs which were associated with FCS (Biscarini et al., 2010b) were also significantly associated with levels of serum natural antibody (NAb) isotypes IgM and IgG binding KLH (Sun et al., 2013). These variations were mostly reported to be associated with the associative genetic effects on FCS, and few with the direct genetic effect (the genetic effect of the individual's own genotype on its FCS). This suggests that the NAb isotypes titers may not only be related with the susceptibility to be pecked at, but particularly with the propensity to perform feather pecking. In our previous study, NAb isotype titers were reported to be associated with survival of laying hens (Sun et al., 2011). As mentioned previously, severe feather pecking and cannibalism may also induce mortality. It is possible that the NAb isotype is associated with survival by regulating the feather pecking behaviour. Therefore in the second part of the present study, we model an individual's FCS as a function of the NAb isotype titers of the individual and those of its cage mates, to investigate the possible relationship between feather pecking behaviour and levels of NAb isotype IgM and IgG in beak trimmed and non-beak trimmed laying hens.

5.2 Materials and methods

5.2.1 Study population

Female crossbred offspring of two commercial purebred White Leghorn layer lines (male W1 and female WB) were provided by Institut de Sélection Animale (ISA) B.V., the layer breeding division of Hendrix Genetics (Boxmeer, The Netherlands). The two purebred lines and the crossbred show high mortality with intact beaks (Ellen et al., 2008; Peeters et al., 2012). The W1 and WB lines were verified as "high and low natural antibody isotype" lines, respectively (Star et al., 2007; Sun et al., 2011). Uitdehaag et al. (2008) showed that birds of the W1 lines have more severe feather pecking behaviour and feather damage than birds of the WB line (Uitdehaag et al., 2008), whereas Ellen et al. (2008) found a higher mortality in the WB line. Fifty sires of line W1 were randomly chosen and mated with 908 dams of line WB, where dams were nested within sires. Each sire was mated to approximately 18 dams, and each dam contributed on average 3 female offspring, resulting in 2,724 offspring.

5.2.2 Housing and management

All chickens were hatched, sexed and wing-banded in the right wing for individual identification. Only female chicks were kept for this study. Offspring of 25 sires were beak trimmed, whereas offspring of another 25 sires were kept with intact beaks. Chicks were trimmed manually at day old using a hot blade to remove and cauterize the tip of the beak. Hens were allocated to rearing cages randomly with respect to beak trimming, 60 individuals per cage. From 5 weeks of age onwards, the hens were housed with 20 individuals per cage. The cage in which an individual was reared was not recorded. The cage number for each hen was not recorded. At 17 weeks of age, all hens were transported to a high-light intensity laying house with conventional 5-bird cages (44 cm height × 40 cm depth × 55 cm width). Each pair of back-to-back cages shared two drinking nipples. A feeding through was in front of the cages, with a length of 55 cm per cage. After placing the birds in the cages, hen were wing-banded in the left wing as well, to avoid loss of data. There were six rows (three double rows) of cages in the laying house, with corridors in between to allow employees to have access to the cages (Figure 5.1). The outer two double rows consisted of three levels (top; middle, closest to the light; and bottom). The middle double rows consisted of four levels (super top; top; middle, closest to the light; and bottom). Hens were only placed in the top and middle levels. Five hens, which were a mix of half sibs and full sibs were allocated to the

same cage. The hens in each pair of back-to-back cages had received the same treatment regarding to beak trimming and could contact with their back neighbors through the wire mesh. Contact with hens in adjacent cages was impossible because of the closed wall in between. Water and standard commercial layer diet was provided ad libitum. Rearing started with a 9L:15D light scheme and increased 1 hour per week until 16L:8D was reached when the hens were 26 weeks of age. The hens received routine vaccinations for Marek's disease (day 1), infectious bronchitis (day 1, week 2, 10, 12 and 15), Newcastle disease (week 2, 6, 12 and 15), infectious bursal disease (week 3 and 15), turkey rhinotracheitis (week 8 and 18), fowl pox (week 15), chicken anaemia virus (week 15) and avian encephalomyelitis (week 15).

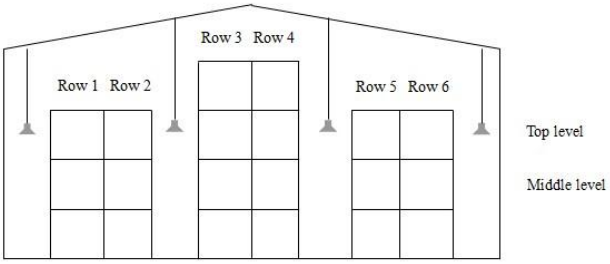

Figure 5.1 The division of the stable, showing the light arrangement, numbers of the cages per row and level (Sun et al., 2013).

5.2.3 Study design

All hens were observed daily from 17 until 83 weeks of age for survival. Hens that died were removed from the cages without replacement. Wing-band number and date of death were recorded. Cause of death was not determined. For each hen, information was collected on survival and survival days. Survival was defined as dead (0) or alive (1) at the end of the study. From this data, survival rate was calculated as the percentage of laying hens still alive at the end of the study. Survival days were defined as the number of days from the start of the observation until either death or termination of the present study, with a maximum of 457 days. At 24 weeks of age, 2 mL blood samples of all birds were taken from the wing vein using the plastic vacuum blood collection tubes containing sodium heparin. The bleeding procedure for each bird was 15 to 30 seconds. The plasma samples were collected after the centrifugation of the blood and used to measure NAb isotype IgM and IgG titers binding KLH. At 53 weeks of age, the individual feather condition of neck, back, rump and belly areas was scored.

5.2.4 NAb isotypes IgM and IgG titers binding KLH

There are no antigens in the environment of laying hens that show immunological cross-reactivity with KLH based on the literature and pilot experiments. Thus, prior exposure or sensitization to this protein is considered unlikely. According to definitions, NAb are immunoglobulins present in animals in the absence of earlier (deliberate) immunization, vaccination or infection (Avrameas 1991). Therefore the antibodies detected in the serum binding KLH were regarded as NAb. Indirect enzyme-linked immunosorbent assay (ELISA) as described earlier (Sun et al., 2011) was performed to measure levels of serum NAb isotypes IgM and IgG binding KLH at 24 weeks of age by the same person on different days. NAb isotypes titers were only measured once for each sample. However, each plate was run with two duplicated positive plasma samples of eight step wised dilutions. The inter-assay CV and intra-assay CV was calculated as 5.1% and 4.2%, respectively.

5.2.5 Feather Condition Scoring

In the present study, feather damage of the laying hens was assessed by evaluating the individual's feather condition at 53 weeks of age of four body areas: neck, back, rump and belly, which are the frequent targets of feather pecking. The scoring was performed by four persons, following the classification of Bilcik and Keeling (1999), as modified by Uitdehaag et al. (2008). In a pilot study, the average correlation between persons performing the scoring was estimated 0.82 for neck, back, and rump, and 0.72 for belly by E. D. Ellen (Wageningen University, Wageningen, The Netherlands, personal communication). There were 6 classes for FCS, ranging from 0 (intact feathers) to 5 (almost all feathers missing), with higher score indicating more damage. The sum of scores of four areas was used as an overall parameter of feather condition. Sum = individual neck score + individual back score + individual rump score + individual belly score (ranging from 0 to 20). Birds that died before 53 weeks of age (252 out of 1,169 non-beak trimmed, and 31 out of 1,555 beak trimmed laying hens) did not receive their FCS.

5.2.6 Data analysis

Descriptive statistical analyses were performed using SAS 9.1.2 (SAS Institute, 2004). Effects were considered significant at the level of $P < 0.05$. A general linear model (GLM) was used to study the differences in FCS between beak-trimmed and non-trimmed birds, and the differences in IgM and IgG titers binding KLH between both groups. The correlations between FCS of four different body areas were estimated

by Pearson product-moment correlation. The average IgM and IgG titer of cage mates of every individual laying hen was also calculated.

5.2.6.1 Variance Components Estimation of FCS

A traditional linear animal model and a direct-associative effect model were used to estimate the variance component of FCS. FCS of beak trimmed and non-beak trimmed laying hens were analysed separately using the GLM procedure of the SAS program (SAS Institute, 2004). To correct for systematic non-genetic differences among observations, factors with $P < 0.10$ from the GLM were included as fixed effects in the model for estimating genetic parameters. Fixed effects for FCS in beak trimmed laying hens were (1) row of cages, (2) level of the cages where the laying hens were located to account for infrastructural effects like light intensity difference (Kjaer and Vestergaard 1999), and (3) person who scored the feather condition. In non-beak trimmed laying hens, only person who scored the feather condition was included as fixed effects.

In order to compare genetic parameters for FCS between beak trimmed and non-beak trimmed laying hens, a bivariate model was used by treating FCS as different traits for both populations.

(1) Traditional Linear Animal Model

Genetic parameters of FCS were first estimated using a traditional linear animal model as implemented in the ASReml software package (Gilmour et al., 2006):

$$\begin{bmatrix} \boldsymbol{y}_1 \\ \boldsymbol{y}_2 \end{bmatrix} = \begin{bmatrix} \boldsymbol{X}_1 & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{X}_2 \end{bmatrix} \begin{bmatrix} \boldsymbol{b}_1 \\ \boldsymbol{b}_2 \end{bmatrix} + \begin{bmatrix} \boldsymbol{Z}_{1\_D} & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{Z}_{2\_D} \end{bmatrix} \begin{bmatrix} \boldsymbol{a}_{1\_D} \\ \boldsymbol{a}_{2\_D} \end{bmatrix} + \begin{bmatrix} \boldsymbol{V}_1 & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{V}_2 \end{bmatrix} \begin{bmatrix} \boldsymbol{cage}_1 \\ \boldsymbol{cage}_2 \end{bmatrix} + \begin{bmatrix} \boldsymbol{e}_1 \\ \boldsymbol{e}_2 \end{bmatrix} \quad (1),$$

where subscript 1 indicates beak trimmed laying hens and subscript 2 indicates non-beak trimmed laying hens; $\boldsymbol{y}$ is a vector of individual sum of FCS at 53 weeks of age, $\boldsymbol{b}$ is a vector of fixed effects, with incidence matrix X linking FCS to fixed effects; $\boldsymbol{a}$ is a vector of usual breeding values, with incidence matrix $\boldsymbol{Z_D}$ linking FCS to the breeding value; $\boldsymbol{cage}$ is a vector of independent random cage effects; $\boldsymbol{V}$ is an incidence matrix linking observations to random cage effects; $\boldsymbol{e}$ is vector of random residuals. The direct genetic (co)variance structure was:

$$\mathrm{var}\begin{bmatrix} \boldsymbol{a}_{1\_D} \\ \boldsymbol{a}_{2\_D} \end{bmatrix} = \begin{bmatrix} \sigma^2_{A_{1\_D}} & \sigma_{A_{12\_D}} \\ \sigma_{A_{12\_D}} & \sigma^2_{A_{2\_D}} \end{bmatrix} \otimes \boldsymbol{A},$$

where $\sigma^2_{A_{1\_D}}$ is the direct genetic variance for beak trimmed laying hens, $\sigma^2_{A_{2\_D}}$ is the direct genetic variance for non-beak trimmed laying hens, $\sigma_{A_{12\_D}}$ is the direct genetic

covariance between beak trimmed and non-beak trimmed laying hens. $\otimes$ indicates the Kronecker product of matrices. ***A*** is the additive genetic relationship matrix generated from a five-generation pedigree. Phenotype variance was calculated as $\sigma_P^2 = \sigma_{A_D}^2 + \sigma_{cage}^2 + \sigma_e^2$. Heritabilities were calculated as $h^2 = \sigma_{A_D}^2 / \sigma_P^2$.

(2) Direct-associative effect model

To estimate genetic parameters for both direct and associative effects, the following model extended from Muir (2005) was used:

$$\begin{bmatrix} \boldsymbol{y_1} \\ \boldsymbol{y_2} \end{bmatrix} = \begin{bmatrix} \boldsymbol{X_1} & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{X_2} \end{bmatrix} \begin{bmatrix} \boldsymbol{b_1} \\ \boldsymbol{b_2} \end{bmatrix} + \begin{bmatrix} \boldsymbol{Z_{1\_D}} & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{Z_{2\_D}} \end{bmatrix} \begin{bmatrix} \boldsymbol{a_{1\_D}} \\ \boldsymbol{a_{2\_D}} \end{bmatrix} + \begin{bmatrix} \boldsymbol{Z_{1\_S}} & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{Z_{2\_S}} \end{bmatrix} \begin{bmatrix} \boldsymbol{a_{1\_S}} \\ \boldsymbol{a_{2\_S}} \end{bmatrix} + \begin{bmatrix} \boldsymbol{V_1} & \boldsymbol{0} \\ \boldsymbol{0} & \boldsymbol{V_2} \end{bmatrix} \begin{bmatrix} \boldsymbol{cage_1} \\ \boldsymbol{cage_2} \end{bmatrix} + \begin{bmatrix} \boldsymbol{e_1} \\ \boldsymbol{e_2} \end{bmatrix} \quad (2),$$

where the vectors and incidence matrices correspond to those in the traditional linear animal model; $\boldsymbol{Z_S}$ is an incidence matrix linking an individual's FCS to its cage mates' breeding value vector. $\boldsymbol{a_S}$ is a vector of social breeding values for all cage mates. The direct-associative genetic (co)variance structure was:

$$\mathrm{var}\begin{bmatrix} \boldsymbol{a_{1\_D}} \\ \boldsymbol{a_{2\_D}} \\ \boldsymbol{a_{1\_S}} \\ \boldsymbol{a_{2\_S}} \end{bmatrix} = \begin{bmatrix} \boldsymbol{\sigma}^2_{\boldsymbol{A_{1\_D}}} & \boldsymbol{\sigma}_{\boldsymbol{A_{12\_D}}} & \boldsymbol{\sigma}_{\boldsymbol{A_{1\_DS}}} & \boldsymbol{\sigma}_{\boldsymbol{A_{1\_D\_2\_S}}} \\ \boldsymbol{\sigma}_{\boldsymbol{A_{12\_D}}} & \boldsymbol{\sigma}^2_{\boldsymbol{A_{2\_D}}} & \boldsymbol{\sigma}_{\boldsymbol{A_{2\_D\_1\_S}}} & \boldsymbol{\sigma}_{\boldsymbol{A_{2\_DS}}} \\ \boldsymbol{\sigma}_{\boldsymbol{A_{1\_DS}}} & \boldsymbol{\sigma}_{\boldsymbol{A_{2\_D\_1\_S}}} & \boldsymbol{\sigma}^2_{\boldsymbol{A_{1\_S}}} & \boldsymbol{\sigma}_{\boldsymbol{A_{12\_S}}} \\ \boldsymbol{\sigma}_{\boldsymbol{A_{1\_D\_2\_S}}} & \boldsymbol{\sigma}_{\boldsymbol{A_{2\_DS}}} & \boldsymbol{\sigma}_{\boldsymbol{A_{12\_S}}} & \boldsymbol{\sigma}^2_{\boldsymbol{A_{2\_S}}} \end{bmatrix} \otimes \boldsymbol{A}$$

where $\boldsymbol{\sigma}^2_{\boldsymbol{A_{1\_D}}}$, $\boldsymbol{\sigma}^2_{\boldsymbol{A_{2\_D}}}$, and $\boldsymbol{\sigma}_{\boldsymbol{A_{12\_D}}}$ are the same as in the traditional linear animal model. $\boldsymbol{\sigma}^2_{\boldsymbol{A_{1\_S}}}$ is the associative genetic variance for beak trimmed laying hens; $\boldsymbol{\sigma}^2_{\boldsymbol{A_{2\_S}}}$ is the associative genetic variance for non-beak trimmed laying hens; $\boldsymbol{\sigma}_{\boldsymbol{A_{12\_S}}}$ is the associative genetic covariance between beak trimmed and non-beak trimmed laying hens; $\boldsymbol{\sigma}_{\boldsymbol{A_{1\_DS}}}$ is the direct-associative genetic covariance in beak trimmed laying hens; $\boldsymbol{\sigma}_{\boldsymbol{A_{2\_DS}}}$ is the direct-associative genetic covariance in non-beak trimmed laying hens; $\boldsymbol{\sigma}_{\boldsymbol{A_{1\_D\_2\_S}}}$ is the genetic covariance between the direct effect of beak trimmed and the associative effect of non-beak trimmed laying hens; $\boldsymbol{\sigma}_{\boldsymbol{A_{2\_D\_1\_S}}}$ is the genetic covariance between the associative effect of non-beak trimmed laying hens and the direct effect of beak trimmed laying hens. The total heritable variance for response to selection was $\sigma^2_{TBV} = \sigma^2_{A_D} + 2(n-1)\sigma_{A_{DS}} + (n-1)^2\sigma^2_{A_S}$ (Bijma et al., 2007). σ^2_P is the phenotypic variance, $\sigma^2_P = \sigma^2_{A_D} + (n-1)\sigma^2_{A_S} + \sigma^2_{cage} + \sigma^2_e$. *n* is the number of

laying hens kept in the same cage, and n = 5 in the present study. T^2 expresses the total heritable variance relative to the phenotypic variance: $T^2 = \sigma^2_{TBV} / \sigma^2_P$.

Likelihood ratio tests were used to test the significance of the random associative effect in univariate model in beak trimmed and non-beak trimmed laying hens, respectively.

5.2.6.2 Trait-based Approach

To investigate whether NAb can explain variation in FCS among individuals, a trait-based analysis for FCS was conducted by fitting a linear mixed model, following Moore et al. (1997). The fixed effects were the same as those detected in the variance components approach. Therefore, the model for beak trimmed laying hens was:

$$y_{ijkl} = \mu + row_i + level_j + person_k + bx + cage_l \quad (3),$$

where y_{ijkl} is individual sum FCS at 53 weeks of age; μ is the overall mean; row_i is the fixed effect of row of the cage (i = 1, 2, 3, 4, 5, 6); $level_j$ is the fixed effect of level of the cage (j = 1, 2); $person_k$ is the effect of the k^{th} (k = 1, 2, 3, 4) person who scored the feather condition; $cage_l$ is the random effect of cage l, x is the fixed effect of individual IgG or individual IgM or average IgM titers of the cage mates or average IgG titers of the cage mates, b is the estimated parameter for the covariable x . The model for non-beak trimmed laying hens was:

$$y_{ikl} = \mu + row_i + person_k + bx + cage_l \quad (4),$$

where all the terms are the same as those specified in model (3). The sum FCS for beak trimmed and non-beak trimmed laying hens was tested for normality before model (3) and (4) were run with a MIXED procedure of SAS program (SAS Institute, 2004).

5.3 Results

5.3.1 FCS of beak trimmed and non-beak trimmed crossbred laying hens

The average individual FCS of the four body areas (neck, back, rump, and belly) at 53 weeks of age for beak trimmed and non-beak trimmed laying hens is shown in Table 5.1. In both populations, the score for belly was the lowest among the four areas, indicating that the belly area was less pecked at. In contrast, the neck and rump were areas with highest scores, indicating more damage in these areas. The

coefficient of variation (C.V.) ranged from 23% to 48% for non-beak trimmed, and ranged from 31% to 70% for beak trimmed laying hens. This indicated considerable variations for the FCS of both populations. GLM analysis showed that the FCS for different body areas and the sum of the FCS in beak trimmed laying hens was significantly lower than that in non-beak trimmed laying hens, indicating that non-beak trimmed laying hens had more feather damage (Table 5.1).

Table 5.1 Number of observations (n), and average feather condition score (FCS)[1] (± SD) of four body areas and sum FCS[2] for surviving and non-surviving of beak trimmed and non-beak trimmed crossbred laying hens.

Population		N	Neck***	Back***	Rump***	Belly***	Sum***
Non-beak trimmed	Survival[3]	813	3.78±0.91	3.54±1.04	3.76±1.03	2.77±1.31	13.77±3.48
	Non survival[4]	104	3.69±0.85	3.70±1.16	3.92±1.12	2.92±1.44	14.32±4.10
	Total	917	3.70±0.85	3.56±1.05	3.78±1.04	2.79±1.33	13.83±3.55
	C.V. (%)[5]		23	29	28	48	26
Beak trimmed	Survival	1,447	2.97±0.91	2.64±1.20	2.84±1.35	1.79±1.25	10.23±3.80
	Non survival	77	2.94±1.13	2.78±1.17	3.13±1.28	1.86±1.31	10.70±4.02
	Total	1,524	2.96±0.92	2.65±1.20	2.85±1.35	1.79±1.25	10.25±3.81
	C.V. (%)		31	45	47	70	37

*** $P < 0.0001$, which indicates that FCS for beak trimmed and non-beak trimmed laying hens is significantly different

[1] There are 6 classes for FCS, ranging from 0 (intact feathers) to 5 (almost all feathers missing), with higher score indicating more damage.

[2] Sum FCS = individual neck score + individual back score + individual rump score + individual belly score (ranging from 0 to 20).

[3] Survival indicated the animals survived until the end of the observation period (83 weeks of age).

[4] Non-survival indicated the animals died between 53 weeks of age (when feather condition scoring performed) and the end of the observation period (83 weeks of age), as the birds died before were not scored.

[5] C.V. (%), coefficient of variation = (SD / Mean)*100%

Both in beak trimmed and non-beak trimmed laying hens, as expected, the correlation coefficients between the scores of different body areas were positive (Table 5.2). The correlations between the areas which were close to each other, like back and rump, neck and back, were higher than those between the areas which were further away from each other, like neck and belly. The sum of scores of these four body areas was used as the aggregated FCS.

Table 5.2 Pearson correlation coefficients1 between the feather condition scores of four body areas (neck, back, rump, and belly) in beak trimmed (above the diagonal) and non-beak trimmed crossbred laying hens (below the diagonal)

	Neck	Back	Rump	Belly	Sum[2]
Neck	--	0.66	0.44	0.37	0.73
Back	0.65	--	0.71	0.46	0.88
Rump	0.50	0.71	--	0.52	0.85
Belly	0.40	0.60	0.62	--	0.75
Sum[2]	0.73	0.89	0.86	0.83	--

[1] All the correlations were significantly different from 0 ($P < 0.0001$).
[2] Sum = sum of the individual feather condition scores of neck, back, rump, and belly areas.

5.3.2 Genetic parameters of FCS

The estimated genetic parameters for FCS in beak trimmed and non-beak trimmed laying hens, using either a bivariate traditional linear animal model or a bivariate direct-associative effect model, are given in Table 5.3. Using a traditional linear animal model, similar and significant additive genetic variance (σ^2_A) were found in both populations. The proportion of phenotypic variance explained by direct genetic variance was denoted as h^2. The estimated h^2 of FCS was slightly higher in non-beak trimmed laying hens (0.20, SE = 0.06) than in beak trimmed laying hens (0.17, SE = 0.05). Using a direct-associative effect model, direct ($\sigma^2_{A_D}$) and associative genetic variance ($\sigma^2_{A_S}$) for FCS were estimated in beak trimmed and non-beak trimmed laying hens, respectively. Total heritable variance relative to the phenotypic variance (T^2), and genetic correlations between the direct and associative effect (r_{DS}) for FCS were also calculated based on those estimations.

Likelihood ratio tests were used to statistically compare the traditional linear animal model and the direct-associative effect model. In the beak trimmed laying hens, final log-likelihoods as reported from traditional linear animal model and from the direct-associative effect model were -1334.52 and -1332.12, respectively. The test statistics $\chi^2_{2df} = 2\times[-1332.12-(-1334.52)] = 4.8$, which corresponds to $P = 0.09$. In the non-beak trimmed laying hens, final log-likelihoods as reported from the traditional linear model and direct-associative effect model were -2258.99 and -2261.48, respectively. The test statistics $\chi^2_{2df} = 2\times[-2258.99-(-2261.48)] = 4.98$, which corresponds to $P = 0.08$. Therefore, using the common criterion of $P < 0.05$, the

associative effect for FCS was not a significant random effect in both beak trimmed or non-beak trimmed laying hens, and including this random effect did not significantly improve the models. This observation agrees with the standard errors of the estimated associative genetic variance and the direct-associative genetic covariance, which were not significantly different from zero (Table 5.3).

5.3.3 NAb Isotype Titers in beak trimmed and non-beak trimmed laying hens

Table 5.4 shows the average NAb isotypes IgM and IgG titers binding KLH in beak trimmed and non-beak trimmed laying hens. For IgG binding KLH, the titers in non-beak trimmed hens were significantly higher than that in beak trimmed hens. Furthermore, in beak trimmed laying hens, IgG titers in the non-surviving birds were significantly higher than that in the surviving birds. For IgM binding KLH, there was no significant difference between beak trimmed and non-beak trimmed laying hens. There was no significant difference for IgM between the surviving and non-surviving birds within beak trimmed nor non-beak trimmed group. Overall, the IgM and IgG titers were higher in laying hens with higher FCS, although the difference was not significant.

5.3.4 Direct and associative effect of NAb isotypes on FCS

In both populations, the direct effects of IgM and IgG on FCS were not significantly different from zero, which indicated that an individual's FCS was not significantly affect by its own isotype titers (Table 5.5). In non-beak trimmed laying hens, the estimated parameters for average titers of IgM and IgG titers of the focal individual's cage mates were not significantly different from zero. In beak trimmed laying hens, the averaged IgG titers of cage mates was a significant factor for the individual's FCS (P = 0.03). The estimated parameter for averaged IgG was 0.36 (SE = 0.16), which indicated that when its cage mates had higher IgG titers, the individual may have worse feather condition. Averaged IgM titers of cage mates was not (P = 0.83).

Table 5.3 Estimated parameters with standard error from traditional and direct-associative animal model for beak trimmed and non-beak trimmed laying hens

	Traditional linear animal model		Direct-associative effect model	
	Non-beak trimmed	Beak trimmed	Non-beak trimmed	Beak trimmed
Log-likelihood[1]	-	-	2.40	2.49
σ^2_{cage}	6.95 ± 0.78	7.18 ± 0.68	6.14 ± 0.88	6.50 ± 0.75
$\sigma^2_{A_D}$	2.31 ± 0.79	2.16 ± 0.60	1.46 ± 0.85	1.99 ± 0.77
$\sigma_{A_{DS}}$			-0.30 ± 0.30	0.24 ± 0.24
$\sigma^2_{A_S}$			0.36 ± 0.20	0.07 ± 0.15
σ^2_e	2.40 ± 0.52	3.11 ± 0.39	2.34 ± 0.60	3.44 ± 0.38
σ^2_P	10.76 ± 1.08	12.46 ± 0.71	11.39 ± 0.81	12.22 ± 0.71
σ^2_{TBV}			4.84 ± 2.73	5.03 ± 2.15
h^2 or T^2	0.20 ± 0.06	0.17 ± 0.05	0.42 ± 0.24	0.41 ± 0.17
r_{DS}			-0.41 ± 0.40	0.63 ± 1.06
$\sigma_{A_{12\_D}}$	1.41 ± 0.75		1.24 ± 0.89	
$\sigma_{A_{12\_S}}$			-0.05 ± 0.32	
$\sigma_{A_{2\_D\_1\_S}}$			-0.006 ± 0.56	
$\sigma_{A_{1\_D\_2\_S}}$			0.21 ± 0.59	
r_D	0.63 ± 0.29		0.73 ± 0.48	
r_S			-0.33 ± 2.02	
r_T			0.24 ± 1.41	

[1] Log-likelihoods for the direct-associative model are expressed as a deviation from those of the traditional linear animal model. In the traditional linear animal model, $\sigma^2_{A_D}$ is the direct additive genetic variance, σ^2_P is the phenotypic variance, $\sigma^2_P = \sigma^2_{A_D} + \sigma^2_{cage} + \sigma^2_e$, h^2 is the direct heritability, $h^2 = \sigma^2_{A_D} / \sigma^2_P$. $\sigma^2_{A_S}$ is the associative genetic variance, $\sigma_{A_{DS}}$ is the direct-associative genetic covariance. In the direct-associative effect model, the total heritable variance $\sigma^2_{TBV} = \sigma^2_{A_D} + 2(n-1)\sigma_{A_{DS}} + (n-1)^2\sigma^2_{A_S}$ (Bijma et al., 2007). σ^2_P is the phenotypic variance, $\sigma^2_P = \sigma^2_{A_D} + (n-1)\sigma^2_{A_S} + \sigma^2_{cage} + \sigma^2_e$, n is the number of laying hens kept in the same cage, n = 5 in the present study. T^2 is the total heritable variance relative to the phenotypic variance: $T^2 = \sigma^2_{TBV} / \sigma^2_P$. r_D is the genetic correlation between the direct and

associative effect. $\sigma_{A_{12\_D}}$ is direct genetic covariance between beak trimmed and non-beak trimmed laying hens, $\sigma_{A_{12\_S}}$ is the associative genetic covariance between beak trimmed and non-beak trimmed laying hens. $\sigma_{A_{1\_D\_2\_S}}$ is the genetic covariance between the direct effect of beak trimmed and the associative effect of non-beak trimmed laying hens. r_D is the genetic correlation between direct effect of beak trimmed and non-beak trimmed laying hens, $r_D = \sigma_{A_{12\_D}} / \sqrt{\sigma^2_{A_{1\_D}} \sigma^2_{A_{2\_D}}}$. r_S is the genetic correlation between associative effect of beak trimmed and non-beak trimmed laying hens, $r_S = \sigma_{A_{12\_S}} / \sqrt{\sigma^2_{A_{1\_S}} \sigma^2_{A_{2\_S}}}$. r_T is the genetic correlation between total heritable variance beak trimmed and non-beak trimmed laying hens, $r_T = \frac{\sigma_{A_{12\_D}} + (n-1)\sigma_{A_{1\_D\_2\_S}} + (n-1)\sigma_{A_{2\_D\_1\_S}} + (n-1)^2 \sigma_{A_{12\_S}}}{\sqrt{\sigma^2_{A_{1\_D}} + 2(n-1)\sigma_{A_{1\_DS}} + (n-1)^2 \sigma^2_{A_{1\_S}}} \sqrt{\sigma^2_{A_{2\_D}} + 2(n-1)\sigma_{A_{2\_DS}} + (n-1)^2 \sigma^2_{A_{2\_S}}}}$ (Peeters et al., 2012).

Table 5.5 Parameter estimates with standard error and the significant level (P-value) of the fixed effects of individual IgG titers, individual IgM titers, averaged IgG titers of cage mates, and average IgM titers cage mates, on individual feather condition score.

	Non-beak trimmed		Beak trimmed	
Fixed effect	Estimate (SE)	*P*-value	Estimate (SE)	*P*-value
IgG	0.09 (0.07)	0.20	-0.07 (0.06)	0.21
IgM	0.09 (0.10)	0.38	-0.08 (0.08)	0.32
Cage mates IgG	-0.15 (0.19)	0.43	0.36 (0.16)	0.03
Cage mates IgM	0.05 (0.19)	0.80	0.04 (0.18)	0.83

5.4 Discussion

In the present study, we compared FCS in beak trimmed and non-beak trimmed crossbred laying hens. Variance component estimation indicated that there was relevant heritable variation for FCS in both populations using a traditional linear animal model. Using a linear animal model combining the direct and associative effects, there were no significant social genetic effects. A possible link between the NAb isotype titers binding KLH and feather pecking behaviour was also investigated.

Table 5.4 Number (n), and average titers (± SD) of NAb isotypes IgM and IgG binding KLH of beak trimmed and non-beak trimmed laying hens

Population			n	IgM titers	IgG titers
Non-beak trimmed	Total		1,169	8.17±1.07	[a]6.71±1.18
	Survival[1]	Surviving	813	8.17±1.06	6.69±1.16
		Non	356	8.18±1.10	6.77±1.24
	Sum FCS[2]	0-4	6	8.38±1.30	7.92±1.60
		5-8	60	7.93±0.99	6.46±1.13
		9-12	251	8.06±1.10	6.53±1.19
		13-16	377	8.18±1.04	6.81±1.16
		17-20	223	8.40±1.05	6.76±1.07
		NA	252	8.18±1.10	6.77±1.27
Beak trimmed	Total		1,555	8.24±1.05	[a]6.61±1.22
	Survival[1]	Surviving	1,447	8.24±1.05	[b]6.59±1.22
		Non	108	8.30±1.03	[b]6.86±1.16
	Sum FCS[2]	0-4	89	8.30±1.06	6.35±1.42
		5-8	436	8.23±0.97	6.58±1.24
		9-12	594	8.20±1.09	6.62±1.19
		13-16	308	8.30±1.10	6.64±1.22
		17-20	97	8.32±0.98	6.80±1.19
		NA	31	8.19±1.00	6.59±1.13

[a] The total IgG titers in beak trimmed and non-beak trimmed laying hens is significantly different with $P < 0.05$.
[b] The IgG titers for the survived and non-survived beak trimmed laying hens is significantly different with $P < 0.05$.
[1] Surviving indicated the animals survived until the end of the observation period (83 weeks of age), non-surviving indicated the animals died between 24 weeks of age and the end of the observation period.
[2] Sum FCS = the sum of feather condition score of four body areas.

5.4.1 FCS in beak trimmed and non-beak trimmed laying hens

Beak trimming is the removal of the tip of the beak of a bird. This treatment is performed as part of an overall strategy to reduce feather pecking and cannibalism, especially in laying hens. In the present study, the feather condition in beak trimmed birds was significant better than that in non-beak trimmed laying hens (Table 5.1). Thus, as expected, beak-trimming reduces feather damage. The variation of FCS was, however, larger than that in non-beak trimmed laying hens. This indicated that beak trimmed laying hens still have feather damage problems due to different extent of feather pecking behaviour. Beak trimming only reduces the mortality instead of preventing the feather pecking propensity.

Among the four body areas, the belly received the least feather damage, while the neck and rump received the most. Similar patterns were also found in non-beak trimmed purebred laying hens (Biscarini et al., 2010b). Bright et al. (2006) also found that the rump area of free-ranged laying hens were most damaged. The fact that back and rump are the areas most exposed to other cage mates could be an explanation. However, in laying hens raised in floor pens, Bilcik and Keeling (1999) observed that the belly region became denuded first. The difference may be caused by the difference in housing systems. Both in beak trimmed and non-beak trimmed laying hens, the Person correlations between the scores of different body areas were positive and high, especially between the areas which were close to each other, back and rump, for example (Table 2). Feather condition scoring as a behavioural measurement at the individual level is a labour-intensive and time-consuming work. Given the extent of damage and correlations between the FCS for different body areas, it may be efficient and sufficient to only score one representative body area, like back or rump.

The survival of beak trimmed laying hens was significantly higher than non-beak trimmed laying hens (Sun et al., 2013). This suggests that severe feather damage maybe a causing factor for the mortality afterwards. However, neither in the beak trimmed nor in the non-beak trimmed laying hens, a significant difference was detected for the FCS between non-surviving and surviving birds. This might rest not only on the limited number of non-surviving hens after 53 weeks of age (the age for feather scoring), but also on the fact that the laying hens died before 53 weeks were not scored (in non-beak trimmed laying hens, a high death rate was observed from 18 to 26 weeks of age, and around 35 weeks of age (Sun et al., 2013)). Unfortunately, these hens did not receive a FCS. Feather damage due to feather pecking cumulates over time. Feather pecking has been observed as early as one

day after hatching (Roden and Wechsler 1998) with sudden increases of cannibalism frequencies in the brooding period of 4 to 11 weeks of age (Hughes and Duncan 1972), and around the onset of egg laying at 20 weeks of age (McKeegan and Savory 1998). It could be speculated that the animals that died before the scoring had a poor feather condition. Peeters et al. (2012) detected a substantial associative effect for survival days in non-beak trimmed laying hens. This together with our analysis on FCS supports the argument that severe feather pecking contributed to the mortality in non-beak trimmed laying hens.

5.4.2 Direct and associative effect for FCS

Similar to other interaction phenotypes, like survival days in laying hens (Ellen et al., 2008; Peeters et al., 2012), FCS of an individual hen is affected by both the individual and its conspecifics when kept in groups. Ignoring the social interaction among individuals which generate additional heritable variation may result in biased genetic parameters estimation. In the present study, we estimated the genetic parameters for FCS in beak trimmed and non-beak trimmed laying hens, using the traditional linear animal model, and a model combining the direct and associative effect, respectively. Using the traditional linear model, the heritability (h^2) for FCS in non-beak trimmed laying hens was estimated to be 0.20 (SE = 0.06), and in beak trimmed laying hens was estimated to be 0.17 (SE = 0.05). Using a direct-associative effect model, the estimated associative genetic variance was substantial, causing the total heritable variation (T^2) to be 2-fold greater than ordinary heritability (Table 5.3). Nevertheless, the estimated associative genetic variance was not significantly different from zero. This also agrees with the results from the likelihood ratio test for the significance of random associative genetic effect (P = 0.09 in beak trimmed birds, and P = 0.08 in non-beak trimmed birds). Hence, our results suggest that associative genetic effects may be important in those populations, but lacked the statistical power to accurately estimate those effects, probably due to a limited number of records (Table 5.1). T. Brinker (Wageningen University, Wageningen, The Netherlands, personal communication) showed that social effects had a substantial effect on the total heritable variation of FCS in purebred non-beak trimmed laying hens (n = 6,276 and 6,916 for two purebred layer lines). Using large data sets, Ellen et al. (2008; n = 3,988 to 6,916 for different purebred layer lines) and Peeters et al. (2012; n = 15,012 for crossbred laying hens) found large and strongly significant social effects on survival time in non-beak trimmed laying hens. However, a suggestive heritable variation from the associative effect for FCS was still indicated from the comparison of analysis with

two models in the present study. Ignoring the associative effect and the genetic correlation between direct and associative effect may induce underestimation of heritable variation for FCS and inappropriate breeding strategies for less feather damage in laying hens. Behavioural measurement on individual level is a real effort. Hence, our results also illustrate the difficulty of collecting sufficient data to accurately estimate genetic parameters for behavioural traits. As we discussed before, to get proper estimation for genetic parameters for FCS, enlarging the numbers of birds involved maybe more valuable than scoring for multiple body areas, because of the high and positive correlation between the FCS of closely-located body areas. Further improvement of statistical power may come from optimizing the cage composition, as results in Bijma (2010) indicate that the standard error of the estimated associative genetic variance is minimized when each cage consist of members of two families, each family contribution half.

5.4.3 Direct and associative effect of NAb isotype IgM and IgG titers binding KLH on FCS

Several studies showed links between feather pecking and immune system. NAb is claimed to be an important parameter of the immune system. To investigate the possible relationship between receiving feather pecking and NAb isotype titers (direct effect of individual NAb on individual FCS), and the relationship between performing feather pecking and NAb isotype titers (associative effect of individual NAb on cage mates' FCS), a mixed model with either the focal individual's or cage mates' average isotype titers as fixed effects was fitted for FCS of the individual in beak trimmed (model 3) and non-beak trimmed laying hens (model 4), respectively.

In both populations, the direct effects of IgM and IgG for individual FCS were not significant (Table 5). This indicated that the individual's own isotype titers may not affect its FCS, although Biscarini et al. (2010a) detected a link between receiving feather pecking and the individual's innate and adaptive immune parameters.

A link between performing feather pecking and the immune parameters was detected (Buitenhuis et al., 2004; Parmentier et al., 2009; Biscarini et al., 2010b; Hughes and Buitenhuis, 2010; Brunberg et al., 2011). In non-beak trimmed laying hens, significant associative effect of NAb titers on feather damage was not detected. However, in beak trimmed laying hens, the parameter estimates for average titers of IgG of the cage mates was 0.34 (SE = 0.16, P = 0.03). This indicated that when the cage mates have higher IgG titers, the individual may have higher suffer from more feather damage. However, multiple hypothesis testing will increase the false positive results. As a statistical method used to correct for

multiple comparisons, false discovery rate (FDR) adjusted P-value was 0.43. This suggested that the relationship between the individual FCS and the average IgG titers of its cage mate may need to be replicated for further confirmation. The relationships were also fitted for the two populations together, by adding beak treatment as an extra fixed effect. Still the relationships were not significant (data not shown).

In our previous study about the relationship between NAb isotype titers and survival in laying hens, NAb isotypes especially IgM was shown to be a protective factor for health-related survival (Sun et al., 2011), and therefore a promising trait to be bred for higher survival of the population. However, the non-significant relationship between NAb isotype titers and individual FCS, the non-significant relationship between NAb isotype titers of the cage mates' and the individual FCS as shown in the present study suggest that the improvement of individual NAb levels does not result in more feather damage.

5.5 Conclusions

To the best of our knowledge, this is the first time that FCS of the laying hens was modelled by direct and associative effect model, and the first time that the direct and associative effects of NAb isotype titers on individual FCS were investigated. The estimated associative genetic variance for FCS was substantial, but not significantly different from zero, probably due to the limited number of records. Results suggested, however, that including associative effects in the model, both in the beak trimmed and non-beak trimmed laying hens, is important to estimate genetic parameters for FCS. Although the effects of immunity on feather pecking behavior were suggested by several studies. NAb isotypes titers did not show significant direct effects or associative effect for individual FCS in the present study. However, further studies are needed to confirm the suggestive relationship between IgG titers and feather pecking.

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6

General discussion

6.1 General introduction

Breeding goals indicate the direction of a breeding program, or in other words in a breeding goal, the traits to be improved are specified. During the past decades, animal breeders have made great genetic improvement in egg production efficiency of laying hens: on average +2.3 eggs per year from 1990 to 2008 (personal communication with Frans van Sambeek from Hendrix Genetics). However, market requirements and social concerns about the products are changing. New legislations in this industry arises accordingly (van Horne and Achterbosch, 2008). Worldwide, but especially in Europe, poultry industry is undergoing substantial changes such as a ban of battery cage housing systems and beak trimming. The alternative floor housing systems, may facilitate the spread of infectious diseases, while the ban of beak trimming can contribute to higher mortality due to feather pecking and cannibalism. Furthermore, given the growing concern about developing of microbes in humans resistance to antibiotics, abundant use of antibiotics in poultry will either be prohibited or restricted (www.bloomberg.com/news/2012-01-04/antibiotic-use-restricted-in-cattle-swine-poultry-by-u-s-regulators.html). These changes or challenges further emphasize the importance of disease resistance and survival in laying hen breeding goals next to maintaining egg production and feed efficiency. Since it takes several years before considerable changes are visible at the commercial population level, breeding goals of the livestock therefore should look ahead, and take both current and future market demands and the consequence of upcoming legislation into account.

Different from the classical production traits that are already included in the breeding goals (e.g. egg number, egg weight, and shell strength), (general and specific) disease resistance, and survival are difficult and expensive to measure. Therefore, breeding the laying hens which are favorable for farmers and consumers requires substantial investment in data collecting and technology. Health can be implemented in the breeding goals just like production traits by (1) defining proper traits which are associated with animals' health status and survival, and (2) understanding the genetic architecture and biological principles underlying the traits, which may support selection. These two points are also the main aims of the present thesis.

6.2 General disease resistance or specific disease resistance?

There are many diseases of great concern that cause substantial economic losses to the poultry industry globally, including viral infections such as Newcastle Disease (NCD) (Irvine et al., 2011), Marek's Disease (MD) (Biggs and Nair 2012), Infectious Bursal Disease (IBD) (Toro et al., 2009), and bacterial infections such as colibacillosis (Goren 1991). In addition, some zoonoses like avian flu (Ungchusak et al., 2005), Salmonella (Loharikar et al., 2013), and Campylobacter infections (Fearnley et al., 2008) severely threaten human health. Preventative vaccine programs and antibiotics are used worldwide to prevent or control diseases in poultry flocks. However, various pathogens developed resistance against drugs and antibiotics (Carraminana et al., 2004). Different individuals respond differently to vaccinations. Vaccines are never 100% protective, and achieving 80% protection in a vaccinated population is considered an effective vaccine (Kohler et al., 2003). For some infectious diseases like colibacillosis, vaccination is not widely practiced because of the large variety of serogroups (Dho-Moulin and Fairbrother 1999). Vaccination of chickens also causes a decrease in the rate of skeletal muscle protein deposition (Hentges et al., 1984). Furthermore, vaccination and drug treatment often require a considerable amount of labor and are relatively expensive as compared to the profit on eggs. From an economical as well as social point of view, reduction in usage of antibiotics and vaccines is a necessary step. As such strategies for protection of the birds from diseases suffer from limitation, breeding laying hens with internal genetic merit for maintaining health is therefore highly desired by the future egg-production industry.

The basic definition of health is the absence of disease (Gunnarsson 2006) and other physiological disorders. Because of the basic function of animal protein production, different from pet animals or wild animals, healthy livestock can be defined as animals that are resistant to diseases, need less drug treatment, and live a longer productive life. Survival is highly related with the ability of the animals to cope with various diseases or other negative environmental disturbances. Therefore survival is the direct reflection of animal health status or homeostasis. The most prominent line of defense against pathogens is provided by the immune system. Environmental factors such as general management, hygiene measures, nutrition, medication, and vaccination schemes have a big influence on -and can modulate- the immune system. They are therefore important components of comprehensive disease prevention and control programs in the modern animal production industry. Because of the complexity of immunity, much slower progress in time is made for genetic improvement. However, genetic improvement of immunity has permanent and cumulative effects in a breeding population, and is a

valid complement to traditional disease management strategies like vaccinations and therapeutic treatments, especially for the diseases where no effective vaccination is available. Therefore, breeding for disease resistance is a fundamental method of improving the health and survival of livestock (Lamont 1998b).

As first distinguished in plants (van der Plank, 1968), but also generalized later in animals including poultry, there are two major types of disease resistance namely specific disease resistance and general disease resistance (Gavora and Spencer 1978). They are different in mechanism as well as genetic background. Specific disease resistance can be defined as resistance against a single or a limited group of related pathogens, normally controlled by a single major gene or a small number of related genes. For some specific disease, which is also the major threat for survival in a species, breeding for specific disease resistance is feasible and essential to improve survival. For example, infectious pancreatic necrosis (IPN) is a severe viral disease of salmon fish. A single gene which is responsible for 70% percent of variation for the resistance characteristic and animals with the resistance genotype can be selected based on genetic information (Houston et al., 2008).

Specific disease resistance mechanisms seem to be the most targeted and effective. However, in chickens, there are various major infectious diseases, viral or bacterial in nature. Great efforts have also been spent on studying the underlying mechanisms for different diseases. Improvement in resistance against each of these diseases would require a large program of testing for resistance and devotion of a significant portion of selection pressure to this purpose. Furthermore, the expense for the challenge experiments required for breeding for specific disease resistance purpose is high (valuable breeding animals and equipment). Challenge trials also present bio security risks. Studies also indicated that the animals which are resistant to one pathogen are not always resistant to other pathogens (Carson 1951; Calnek et al., 1975; Lamont 1998a), because of the different pathogenicity mechanisms involved. The chicken major histocompatibility complex (MHC), also designated as the B complex, is a group of closely linked polymorphic regions: BF (class I), BL (class IIβ), and BG (Ig-superfamily genes) (Kaufman et al., 1999). MHC cell-surface proteins play an important role of regulation of cellular communication (Dietert, 1987), are known for strong genetic associations with disease resistance and susceptibility to many pathogens including MD virus (Dalgaard et al., 2003), Rous sarcoma tumour virus (Bacon et al., 1981; Plachy and Benda 1981), autoimmune thyroiditis (Kuhr et al., 1994) and Salmonella (Cotter et al., 1998) and so on. However, no single MHC haplotype performs optimally in all genetic backgrounds in response to all important diseases (Lamont 1998a).

Given the different mechanisms involved for disease resistance against various pathogens, it becomes impractical to include them all in a breeding program. However, if breeding for disease resistance is highly specific, for example, limited to a strain of *E. coli* (Cavero et al., 2009), it may protect animals from the strain under study, but provide little or no protection for variants of the same *E. coli* strain. Additionally, (pathogenic) viruses and bacteria usually have high rates of mutation therefore the potential to evolve rapidly (Suzuki et al., 2007; Su et al., 2008). According to the present laying hen breeding programs, it takes 4 years to permit the transmission of genetic improvement from the nucleus breeding populations (where all selection takes place) to the crossbred progeny (commercial layers) (Cavero et al., 2009). It is difficult and even impossible to predict 4 years beforehand how the new strain of pathogens like *E. coli* may mutate or evolve. The breeding response from the previous selection for specific disease resistance can thus be outdated after 4 years. This is similar to the difficulty in developing vaccines effective for all strains.

In contrast to specific disease resistance, general or non-specific resistance to disease could be defined as the ability to resist any alteration of the state of the body by external causes including pathogens and stress, which interrupt or disturb proper performance (Gavora and Spencer 1978). In other words, the general resistance grants the animal with the ability to cope with a wider spectrum of pathogens and stress. The general resistance also indicates the ability of animals to recover from the diseases or disorder (Gavora and Spencer 1978).

Commercial laying hens are now reared for about 80 to 90 weeks for egg production. To maximize the usage of housing facilities, it is the aim of some laying hen breeding companies to extend this period to be as long as 100 weeks for Brown egg layers and 110 weeks for White egg layers by 2020 (www.worldpoultry.net/Breeders/General/2011/3/Breeding-for-500-eggs-in-100-weeks-WP008564W/). This aim also challenges the laying hens' ability to cope with prolonged disease pressure and other disturbances. Laying hens receive standard routine vaccinations in the first 16 weeks of their life to prepare their specific immune response system well for the most common diseases including MD, IBD, and NCD, and survive the laying period. Still, 3 to 20% (depended on lines) of the purebred laying hens (non-beak trimmed) will not survive till the end of laying period (Star, 2008). In commercial crossbred White leghorns, a survival of 89.6% was observed (unpublished data from Esinam Amuzu). This indicated that the vaccination to activate the specific immunity does not provide the animals with full protection. Therefore, except for more complex vaccination procedure, the general

disease resistance is also crucial for the laying hens. Except for diseases, environmental stress such as improper ventilation, temperature, light, and dust burden, stress from peak egg production also may contribute to disorders like respiratory system diseases in laying hens.

There are wide discussions between the immunologists and breeders about what is more important, specific disease resistance or general disease resistance for improved health and survival in poultry. Specific and general resistance supplement each other. Only focus on the specific disease resistance will not fulfill the present or future requirement for general healthy animals. Ochsenbein and Zinkernagel (2000) argued that non-specific immunity (innate immunity) facilitates and adds to specific immunity. NAb may play an important role in enhancing survival of the host by providing early resistance against infection. Therefore, breeding for general disease resistance can be an alternative or additional way to breed for disease resistance. However, potential of improvement of innate immunity for health and survival has not been investigated in great detail. Traits that characterize such general disease resistance (indicator traits) should be defined first.

6.3 Potential of NAb isotype titers as indicators for general disease resistance

The immune system is complex, involving many components playing different roles. For the general disease resistance, some innate immune components, for example, natural resistance associated macrophage protein 1 (NRAMP1) (Zaharik et al., 2002; Liu et al., 2003; Hu et al., 2011), interferon-gamma (IFN-γ) (Yun et al., 2000; Zhou et al., 2002), and Toll-like receptor (TLRs) (Bernasconi et al., 2003; Temperley et al., 2008; Kannaki et al., 2010) attracted much research attention. NAb, which are the antibodies present in the circulation of normal healthy individuals in the absence of a deliberate antigen exposure like vaccination (Avrameas 1991), is an important humoral part of innate immunity. NAb were reported but only regarded as the “background” and “noise” of the immune system before (Korver et al., 1984; Vollmers and Brandlein, 2006). Now it becomes more widely acknowledged for their multiple functions in immunology (Ehrenstein and Notley 2010). NAb are emerging as a potential trait which may be used to characterize general disease resistance and predictive for survival, according to the comparison with other immune parameters including SpAb binding NCD vaccine and haemolytic (classical and alternative) complement activity (Star et al., 2007).

Three NAb isotypes were identified in chickens, named IgM, IgG (IgY), and IgA. The levels of IgA antibodies were not studied since levels of IgA binding KLH in serum are very low. IgA is important in lungs and intestinal tract, providing protective roles against pathogens (Mestecky et al., 1999). This thesis focused on IgM and IgG, their relationship with survival of laying hens, genetic background, and their relationships with other behaviour or growth-related traits. The potential of NAb isotype titers as indicators for general disease resistance which can be implemented into a breeding program for improving general health and survival of laying hens are further discussed in the following sections of this chapter.

6.3.1 Measuring NAb isotypes

Compared with measurement of SpAb responses to a vaccination (e.g. NCD and MD) or antigenic stimulation (e.g. *E. coli* and SRBC), NAb isotype titers in the serum samples are easy and fast to measure on large scale, at a low cost. In addition, blood sample was only collected once for measuring NAb titers, thus less stress from stimulations was caused for the animals which may have large effect on production performance.

In this thesis, antigen KLH was used as a model antigen to measure NAb isotype titers. Prior exposure or sensitization to KLH is considered unlikely for chickens. Except for KLH, NAb binding many different self-antigens and exogenous antigens in chickens were also detected (likely representing NAb idiotypes) (Parmentier et al., 2004). The “idiotype” refers to the unique antigenic determinants recognized by individual antibody molecules on molecules of identical specificity, and reflects the antigen-binding fragment (Fab) of the antibody molecule. It is important to note that enhanced levels of one NAb idiotype may be accompanied by decreased levels of other NAb idiotypes. The latter was suggested (Haghighi et al., 2006) and strengthened (Berghof et al., 2010) earlier. Yet maybe different antigens other than KLH can be identified, which represent superior idiotypes in relation to definition of NAb populations and indicative of greater health potential. KLH is an extremely large, heterogeneous glycosylated protein. Thus it is difficult to distinguish which part of KLH molecular is recognized by an antibody. Using alternative pure proteins can be more specific. For example, proteins resulting from apoptotic processes like actin, ubiquitin, other cellular compartment specific glycoproteins, a lipids might even present better (cryptic) NAb definitions. Shaw et al. (2000) found that NAbs with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity (Shaw et al., 2000).

In previous studies in our lab, including this thesis, “titer”, which is calculated based on a mathematical conversion of Logit-log method (Wild 2005), was used widely as a way of expressing relative concentration of the antibody. The difference of 1 titer unit equals a 2 times real concentration difference. Earlier, Ploegaert (2010) recommended developing the ELISA test from a titer determination further into the detection of the absolute amounts of antibodies present in samples for future use. Although this may face some technical challenges, measuring the absolute amounts of NAb isotypes can make the results from different populations of different time points and different studies better comparable. Furthermore, the absolute amounts of antibodies between animals or multiple samples of the same animals at different time points are more accurate and sensitive than the titers difference.

Furthermore, in the previous studies about NAb in chickens, “total NAb” titers were studied without distinguishing different immunoglobulin isotypes. Biologically, measurement of NAb isotype titers is more accurate. In the ELISA test for measurement of total NAb titers, rabbit-anti-chicken IgG H+L (RACh/IgGH+L/PO; Bethyl Laboratories, Texas, U.S.A.) was used as second antibody (Parmentier et al., 2004; Star et al., 2007). In chickens, the isotypes IgG, IgM, and IgA share the same light chain, but carrying different heavy chains. The antibody with the same heavy chain of IgG (only IgG) and the same light chain of IgG (IgG, IgM, and IgA) will be detected using this second antibody. Therefore, the exact component measured is not clear. Fc (Fragment, crystallizable) region of the antibody includes the isotype-specific heavy chain. In this thesis, rabit-anti-chicken IgG-Fc antibody (RACh/IgG/Fc/PO), and rabbit-anti-chicken IgM antibody (RACh/IgM/PO) were used as second antibody for the measurement of IgG and IgM isotype titers, respectively. Compared with the measurement of total NAb titers, the measurement for NAb isotype is more specific. Therefore, NAb isotypes are technically and biologically more appropriate as selection criteria than the total NAb. Besides the technical advantage of measuring NAb isotypes over the total NAb levels, more evidence will be listed to support this in the following discussion sections.

6.3.2 Genetic variation of NAb isotype titers

One of the prerequisites for the traits to be implemented in the breeding programs is the existence of genetic variation. Without genetic variation, there is no possibility for the improvement of the traits by selection. Estimation of genetic parameters for the traits of interest is also important for predicting the selection response, setting up correct breeding program and estimating individual breeding

value. Lower heritability suggests that greater environmental components were present and selection progress will be slow. On the contrary, higher heritabilities are indication for larger selection response.

In chapter 2, we determined the NAb IgM and IgG isotype titers from the serum samples of 12 purebred layer lines (six White Leghorn lines and six Rhode Island Red lines) at 20, 40, and 65 weeks of age, respectively. These animals were housed in standardized commercial environmental conditions. However, significant differences in IgM and IgG titers among breeds, lines, and among individuals within lines were observed. These differences demonstrate that the humoral innate immunity is determined partly by genetic factors. On average, the coefficient of variation for IgM was lower than that for IgG. Heritabilities for NAb isotype titers were then estimated in purebred (chapter 3) and crossbred laying hens (chapter 4), respectively. The presence of genetic variation in NAb isotype titers between laying hens was proven. In the purebred laying hens, the heritabilities for IgM titers at different ages (20, 40, and 65 weeks of age) were around 0.4, while for IgG they varied from 0.14 for IgG40 (IgG titers at 40 weeks of age) to 0.31 for IgG20 (IgG titers at 20 weeks of age) (chapter 3). In the crossbred laying hens, the heritabilities for IgM and IgG titers at 24 weeks of age was 0.26 and 0.21, respectively (chapter 4). Both NAb isotypes displayed moderate to high heritabilities, indicating that variability of NAb isotype titers is partly due to genetic differences, and that mass selection may be effective.

The estimated heritabilities for NAb isotype binding KLH were higher than those for total NAb titers by Wijga et al. (2009). This can be explained by the fact that the NAb isotype IgM and IgG are factually existing in the animals serum, while the total NAb (in serum) titer is a more comprehensive but vague indicator for humoral innate immunity including IgM and IgG (and less IgA) isotypes. Furthermore, the isotype IgM is more heritable than IgG (chapter 3). These results indicated that IgG level might be very dependent on presence of environment triggers. In contrast, differences in IgM titers are affected to a large extent by genetic factors. This is in accordance with the speculation that the production of the IgM isotype is driven by endogenous (auto) antigens and largely unaffected by external antigens (Haury et al., 1997). B cells can change the isotype of the antibody they produce (from IgM to IgG or IgA) by isotype class-switch after being activated (Market and Papavasiliou 2003). The observed higher heritabilities for IgM than IgG agree with the observation that IgM NAb are naturally present regardless of environmental factors, while their transformation into IgG NAb likely occurs in response to the effect of stimulating factors affecting B cells. NAb isotypes titers show an

advantage over total NAb titers because of the higher heritabilities, especially for the IgM isotype.

A maternal effect is a situation where the phenotype of the offspring is not only determined by its genotype and the environment it experiences, but also by the genotype and/or environment of its mother (Willham 1972). In birds like American Coot, mothers may pass down hormones in their eggs that affect an offspring's growth and behaviour, and social interactions (Reed and Vleck 2001). Maternal antibody also can be transferred through the egg yolk (IgG) in the ovary and albumen (IgM and IgA) in the oviduct (Brown et al., 1989; Hammouda et al., 2012). These antibodies provide the young chicks with protection against pathogens before their own immune system is fully developed. In addition to the passive protection transferred to the chick, maternal immune factors may affect the development of the juvenile's immune system (Zinkernagel 2003; Lemke et al., 2009). In humans, the autoantibody IgG isotype repertoires of each mother and baby were very closely related and distinct for each mother-newborn pair (Madi et al., 2009). These studies suggested transfer of maternal immune functions across generations (Grindstaff et al., 2003; Staszewski and Siitari 2010). In laying hens, respectively 15% and 10% of the SpAb and NAb levels variation at 5 weeks of age was attributed to maternal environmental effects, (Wijga et al., 2009).

The maternal effect is important to consider in (1) estimation of genetic parameters: direct heritability can be overestimated when the maternal effect exists but is neglected (Clement et al., 2001; Kruuk and Hadfield 2007), (2) more accurate prediction of breeding values, and (3) usage of these genetic effects in selection, e.g. crossbreeding. Crossbreeding has become a standard practice in poultry breeding programs. In the present project (chapter 3 and 4), because of the population structure, it was not feasible to detect a maternal effect for NAb isotype titers. Therefore, before the breeding program involving selection for NAb isotype titers, I suggest to set up a population that allows estimation of maternal effect.

6.3.3 Relationship between NAb isotype titers and breeding goal

Besides the genetic variation for the traits to be implemented into the breeding program, another prerequisite for traits is the correlations with the breeding goals Therefore, the relationship between NAb isotype titers and survival need to be investigated.

There are two lines of defense in a bird's immune system: the innate, responding instantly to all antigens, and the adaptive, which will be initiated if the innate

immunity does not succeed in blocking the entry of pathogens or when activated by the innate immunity. Antibodies classically are the prime representatives of adaptive immunity. Different antibodies are highly specific to different antigens, so called idiotype. However, assigned to the innate immune system, NAb supposedly do not increase or adapt (change their idiotype) to antigenic challenge (Nawata et al., 1990). As component of the first line of defense, NAb are indicated to perform crucial homeostatic housekeeping functions in the maintenance of physiological and immunological homeostasis, protecting the body against stress-induced altered self-antigen immunity (Cheng 1998; Lutz 2007; Lutz et al., 2009; Ehrenstein and Notley 2010), as well as induction of a normal adaptive response (Baumgarth et al., 2000). Therefore, a sufficient NAb levels might be crucial for survival of animals.

In chapter 2, the relationships between the NAb isotype titers at 20, 40, and 65 weeks of age and the survival afterwards were investigated using logistic regression. These time points represent birds close to sexual maturity (20 weeks of age), middle age (40 weeks of age), and birds close to the end of the laying period (65 weeks of age), respectively. It is true that birds are most susceptible at the first weeks of their life. The NAb isotype titers were not determined to investigate their relationship with the early survival, because the isotype titers at young age (5 weeks) were too low to be measured accurately (data not shown).

The data indicated that the NAb isotype titers decreased with age based on the three time points in the study (chapter 2). The number of B-1 cells, which likely produce NAb, is not fixed over the life span of an individual. From the neonatal period to adolescence, B-1 cells (in mammals) first increase and then decrease at older age. In human, it is thus tempting to speculate that the increased susceptibility to infection of elderly people may be, in part, due to the decreased number of B-1 cells and the decrease in NAb levels (Kohler et al., 2003). The commercial laying hens reach sexual maturity around 20 weeks of age. Poultry B cells proliferate extensively within the bursal follicles, until the bursa starts to involute as the bird approaches sexual maturity (Jolly, 1915). Therefore, the NAb isotype levels at 20 weeks of age maybe the peak of NAb isotype levels of the birds' lifetime and represent the overall development of the immune system. In the logistic regression analysis in chapter 2, in Rhode Island Red laying hens, odds ratios of 0.56 ($P < 0.001$) for IgM20 were estimated, which means that if IgM20 titers increase by one unit, the relative change in risk of dying during the first period of laying decreases by 44%. The odds ratio for IgG20 was 0.72 ($P = 0.02$), indicating a decrease of 28% in the risk of dying per unit increase in IgG. These

results indicated that isotype, especially IgM titers at 20 weeks of age, was predictive for survival of the laying period afterwards in the purebred laying hens.

Compared with an odds ratio of 0.80 (P = 0.008) estimated for total NAb titers binding KLH in an earlier study (Star et al., 2007), NAb isotype titers are thus more accurate predictors for survival. The odds ratios for IgM and IgG were not the same, either (chapter 2). This further emphasizes the necessity of distinguishing NAb isotypes and studying the diverse isotype-related functions.

Three NAb isotypes are presently identified in chickens, named IgM, IgG, and IgA. All previous studies in chickens as well as this thesis, focused only on IgM and IgG levels in serum or plasma. The levels of IgA antibodies were not studied since levels of IgA binding KLH in serum are very low. In milk samples, IgA was widely present and proved to be functional: heifers without clinical mastitis or high somatic cell count (SCC) history, but with high NAb titers of the IgA and IgM isotypes in their milk show a tendency towards a reduced risk to develop clinical mastitis later in lactation (Ploegaert 2010). IgA is of prime importance for gut health and shaping the microflora population. A large amount of intestinal IgA is either nonspecific or of low affinity for a broad range of antigens (Kroese et al., 1989; Kroese et al., 1993; Thurnheer et al., 2003; 2005). Pathogenic bacterial infections of the intestinal tract are also a major cause of suffering and death of the chickens. The natural IgA located in the gut may play a similar or different role in mucosal immunity as natural IgM and IgG in serum is an interesting area.

6.3.4 Genetic markers associated with NAb isotypes titers

Marker-assisted selection (MAS) was proposed as a method of selecting the animals with superior performance using DNA markers, especially for the traits which are labor-intensive and expensive to measure (for instance, behavioral measurements and immunological parameters). These DNA markers do not necessarily represent causative variations (Fulton 2012). Association studies are necessary before MAS can be applied.

In chapter 3, an association study was performed across lines to detect SNP markers that are closer to the QTL than using a single line, and have the same phase of association in the entire population. Although in chapter 2, only NAb isotype titers at 20 weeks of age were predictive for survival afterwards, the association studies were performed for all traits (IgG and IgM titers at 20, 40, and 65 weeks of age, respectively). Forty-three significant associations between SNPs and isotype titers were detected. The 12 SNPs which were detected for IgM20 and

the 12 SNPs which were detected for IgG20 can be used as markers for selection. The associations between SNPs and mortality could be of interest e.g. by using logistic regression with survival as response variable and SNP genotype as explanatory variable. However, the DNA for genotyping was extracted from the blood sample taken at 40 weeks of age. Animals that died before that age were thus not included in the association analysis. This makes the current data not optimal for associations between SNPs and survival.

Based on the estimated genetic correlations between the traits in the association study (chapter 3), these traits were genetically related with each other. Therefore, except for looking at the associated SNP markers for each trait independently, the detected association pattern was also informative for understanding the relationships of NAb isotypes traits at different time points or the relationships between different isotypes at the same time point. First, different SNPs were detected for IgM and IgG. This might be due to methodological reasons as well as biological reasons. We estimated a positive genetic correlation (0.24, SE = 0.10) between IgM20 and IgG20 which indicates that there are possibilities to improve both traits simultaneously. However, this does not exclude the presence of chromosomal regions with antagonistic effects on IgM20 and IgG20. Furthermore, the genetic correlations between the NAb isotype at older age (40 and 65 weeks) were higher. More SNPs associated with isotype titers at 40 and 65 weeks of age were also found. The genetic correlations between the NAb isotype at young age (20 weeks) and older age (40 or 65 weeks) were lower. Less SNPs were found associated with isotypes at both young and older ages. These results indicate that the genetic backgrounds for developing a functional innate immunity to produce NAb isotypes at young age is different from that for maintaining a stable isotype production until 65 weeks of age. As we discussed before, at 20 weeks of age, the commercial laying hens reach their sexual maturity. That is also the age when the bursa of Fabricius, the organ producing B cells in chickens start to involute. Therefore, the NAb isotype levels at 20 weeks of age maybe the peak of NAb isotype levels of the birds' lifetime and represent the overall development of immune system. This may be an explanation why only isotype titers at 20 weeks of age were predictive for survival.

The association study indicated that some known immune trait-related genes may interact with NAb isotypes. For example, the interleukins (IL) *IL10* and *IL19* were found to have a role in regulating the production of both isotypes. The tripartite motif containing 33 (*TRIM33*) showed significant association with IgG20; heat shock protein 90 kDa alpha (cytosolic), class B member 1 (*HSP90AB1*) affected IgG titers

at older ages. *IL6* was associated with IgM20. These genes may not be the actual genetic regions determining the NAb isotype titers. However, based on their reported functions in literature, we think it is worthwhile to mention some of the listed genes that may have a direct or indirect (network) effect on regulating the isotype titers.

In the present association study for NAb isotype titers, only limited numbers of SNPs were included. The genetic gain from MAS that uses only fewer SNPs in LD with QTL for the interested traits is likely to be small (Dekkers, 2004; Hayes and Goddard 2010). The SNP numbers detected in chickens genome has increased from 2.8 million (Wong et al., 2004) to more than 11 million in 2010 (www.ncbi.nlm.nih.gov/projects/SNP/). These SNPs information provide large numbers of potential markers both for research (QTL mapping, association studies) and commercial application. The rapid reduction in sequencing cost as well as the development of 60K (Groenen et al., 2011) and 600K (Kranis et al., 2013) chicken SNP genotyping array enables the genomic selection, i.e. using a genome-wide panel of denser markers to contributing to the variation in complex traits and predict the genomic breeding value (Meuwissen et al., 2001; Hayes and Goddard 2010). Genomic selection will also have a profound impact on breeding companies about optimizing the breeding programs and improving selection efficiencies for the important traits.

In this genomics era, animals with genetic merit can be selected without knowing the underlying mechanism relating genetic variation and phenotype variation. The chicken is not only a major livestock species but also an important model organism for biological studies. Studies in birds have contributed greatly to the development of immunological understanding. The birds have many immunological mechanisms in common with mammals, but also possess their distinct strategies. Furthermore, because of the potential importance of NAb isotype titers in laying hens as proved in the present thesis, a better understanding of the mechanisms of NAb isotypes is necessary. Genome-wide association study (GWAS) is acknowledged for some advantages, like the power to detect causal variants with modest effects and in defining narrower genomic regions that harbor causal variants (Hirschhorn and Daly, 2005). GWAS and further validation study are therefore indicated to confirm the SNPs detected in the previous study, discover genes or detect causative variant contributing to these traits, reveal the immune pathways that the NAb isotypes are involved, and facilitate their practical understanding in artificial breeding program.

6.3.5 NAb isotypes titers and survival in crossbred laying hens

In chapter 2, we presented the prediction of NAb isotype titers for survival of purebred laying hens. In egg-production industry, commercial laying hens are usually crossbred. Therefore in chapter 4, we investigated the predictive value of NAb isotype titers for survival in crossbred laying hens. A population of crossbred female offspring from purebred W1 (male) and WB (female) lines was used. The offspring of 25 sires were beak trimmed, whereas the offspring of another 25 sires were kept with intact beaks. Different from purebred laying hens, there was no significant association between NAb isotype titers and survival in beak trimmed or non-beak trimmed crossbred laying hens. These inconsistent results request a careful check for the possible reasons, for example, survival difference in different populations, genetic correlations of NAb isotype titers in the purebred and crossbred laying hens.

Selection for improved NAb isotype titers is possible by selection in the purebred lines based on NAb measured in crossbreds, if the genetic correlation between the traits in purebred and crossbred lines is high. Our dataset used in chapter 4 only contained NAb isotype titers measured on crossbred offspring. It was not possible to estimate this genetic correlation. It is, however, important to estimate the genetic correlation before developing a strategy to breed for enhanced NAb titers in crossbred birds.

Survival is highly related with the ability of a living organism to cope with the diseases or other negative environmental disturbances. Therefore it is the direct reflection of animal health status. It is certain that ability to survive is determined by the individual's genetic background. The higher survival of crossbred offspring indicated a heterosis for survival. It is possible that heterosis has a more prominent effect on survival than the NAb isotypes levels in the offspring (chapter 4).

In this thesis, when we talk about survival earlier, we only refer to the health-related survival. However, apparently in poultry especially the laying hens housed in groups, survival or survival days is a so-called interacting phenotype, a trait whose value is not only determined by individual genetic background, but also affected by the behaviour of an individual's conspecifics (Moore et al., 1997), for example, severe feather pecking and cannibalism for the laying hens with intact beaks (Peeters et al., 2012). Using a traditional linear animal model (model 1) and direct-associative effect model (model 2), we could estimate the direct and associative effect for survival days. The traditional linear animal model was

$$\boldsymbol{y} = \boldsymbol{Xb} + \boldsymbol{Za} + \boldsymbol{Vcage} + \boldsymbol{e} \quad (1),$$

where ***y*** is a vector of survival days, ***b*** is a vector of fixed effects, with incidence matrix ***X*** linking survival days to fixed effects; ***a*** is a vector of the usual breeding values, with incidence matrix ***Z*** linking survival days to the breeding value; ***cage*** is a vector of independent random cage effects; ***V*** is an incidence matrix linking observations to random cage effects; e is vector of random residuals. $\mathrm{var}(a) = A\sigma_a^2$, with ***A*** being the additive genetic relationship matrix, σ_a^2 being the genetic variance; $\mathrm{var}(cage) = I\sigma_{cage}^2$, with ***I*** being an identity matrix, σ_{cage}^2 being the cage variance; $\mathrm{var}(e) = I\sigma_e^2$, with σ_e^2 being the residual variance. Phenotype variance was calculated as $\sigma_P^2 = \sigma_a^2 + \sigma_{cage}^2 + \sigma_e^2$. Direct heritabilities were calculated as $h^2 = \sigma_a^2 / \sigma_P^2$.

To estimate genetic parameters for both direct and associative effect, the following model extended from (Muir 2005) was used:

$$\boldsymbol{y} = \boldsymbol{Xb} + \boldsymbol{Z}_D\boldsymbol{a}_D + \boldsymbol{Z}_S\boldsymbol{a}_S + \boldsymbol{Vcage} + \boldsymbol{e} \quad (2),$$

where ***y*** is a vector of individual survival days at 53 weeks of age, ***b*** is a vector of fixed effects, with incidence matrix ***X*** linking survival days to fixed effects; $\boldsymbol{a_D}$ is a vector of the usual breeding values, with incidence matrix $\boldsymbol{Z_D}$ linking FCS to the breeding value; $\boldsymbol{a_s}$ is a vector of the associative breeding values of the individual's cage mates, with incidence matrix $\boldsymbol{Z_S}$ linking survival days to the breeding values of the individual's cage mates; ***V*** is an incidence matrix linking observations to random cage effects; ***cage*** is a vector of independent random cage effects; ***e*** is vector of random residuals. The covariance structures of genetic terms is: $\boldsymbol{var}\begin{bmatrix}\boldsymbol{a_D}\\ \boldsymbol{a_S}\end{bmatrix} = \boldsymbol{C} \otimes \boldsymbol{A}$, where $\boldsymbol{C} = \begin{bmatrix}\sigma_{A_D}^2 & \sigma_{A_{DS}}\\ \sigma_{A_{DS}} & \sigma_{A_S}^2\end{bmatrix}$, indicates the Kronecker product of matrices, $\sigma_{A_D}^2$ is the direct genetic variance, $\sigma_{A_S}^2$ is the associative genetic variance, and $\sigma_{A_{DS}}$ is the direct-associative genetic covariance. The total heritable variance for response to selection was $\sigma_{TBV}^2 = \sigma_{A_D}^2 + 2(n-1) + (n-1)^2\sigma_{A_S}^2$ (Bijma et al., 2007). σ_p^2 is the phenotypic variance, $\sigma_P^2 = \sigma_{A_D}^2 + (n-1)\sigma_{A_A}^2 + \sigma_{cage}^2 + \sigma_e^2$. *n* is the number of laying hens kept in the same cage, and *n* = 5 in the present study. T^2 expresses the total heritable variance relative to the phenotypic variance: $T^2 = \sigma_{TBV}^2 / \sigma_P^2$. The estimated variance components and genetic parameters for survival days in beak trimmed and non-beak trimmed laying hens using two models are presented in Table 6.1.

Table 6.1 Estimate of parameters for survival days with standard error from traditional and direct-associative animal model for beak trimmed and non-beak trimmed laying hens.

	Traditional linear animal model		Direct-associative effect model	
	Non-beak trimmed	Beak trimmed	Non-beak trimmed	Beak trimmed
σ^2_{cage}	7026 ± 875	74 ± 43	6281 ± 923	77 ± 50
$\sigma^2_{A_D}$	768 ± 786	0.0004±0.00002	0.004 ± 0.0002	54 ± 91
$\sigma_{A_{DS}}$			~0.00 ± 0.00	-0.05 ± 15.85
$\sigma^2_{A_S}$			203 ± 134	~0.00 ± 0.00
σ^2_e	10480 ± 725	2032 ± 82	10842 ± 522	1985 ± 113
σ^2_P	18273 ± 950	2105 ± 76	17937 ± 926	2116 ± 81
σ^2_{TBV}			3256 ± 2150	54 ± 89
h^2 or T^2	0.04 ± 0.04	~0.00 ± 0.00	0.18 ± 0.12	0.03 ± 0.04
r_{DS}			-0.00 ± 0.00	0.63 ± 1.06

In the traditional linear animal model, $\sigma^2_{A_D}$ is the direct additive genetic variance, σ^2_P is the phenotypic variance, $\sigma^2_P = \sigma^2_{A_D} + \sigma^2_{cage} + \sigma^2_e$, h^2 is the direct heritability, $h^2 = \sigma^2_{A_D} / \sigma^2_P \cdot \sigma^2_{A_S}$ is the associative genetic variance, $\sigma_{A_{DS}}$ is the direct-associative genetic covariance. In the direct-associative effect model, the total heritable variance $\sigma^2_{TBV} = \sigma^2_{A_D} + 2(n-1)\sigma_{A_{DS}} + (n-1)^2\sigma^2_{A_S}$ (Bijma et al., 2007). σ^2_P is the phenotypic variance, $\sigma^2_P = \sigma^2_{A_D} + (n-1)\sigma^2_{A_S} + \sigma^2_{cage} + \sigma^2_e$, n is the number of laying hens kept in the same cage, n = 5 in the present study. T^2 is the total heritable variance relative to the phenotypic variance: $T^2 = \sigma^2_{TBV} / \sigma^2_P \cdot r_D$ is the genetic correlation between the direct and associative effect.

These results indicated that for the non-beak trimmed laying hens, including the associative effect resulted in a substantially larger heritable variation: increased from 0.04 (SE = 0.04) to 0.18 (SE = 0.12). Most of the increased variation came from the associative effect. In the beak trimmed laying hens, including the associative effect also resulted in an increased heritable variation, whereas, most increase came from the direct effect. These results indicated that in the non-beak trimmed laying hens, survival was more affected by the associative effect, i.e. the individual's survival was largely determined by the behaviour of cage mates. On the contrary, the survival of beak trimmed crossbred laying hens was more determined by the direct effect, i.e. individual's genetic background underlying ability of

keeping health and survival. Therefore, the survival in beak trimmed and non-beak trimmed laying hens are different traits.

In chapter 5, we showed that the individual's NAb isotype titers of both beak trimmed and non-beak trimmed laying hens were not related to feather pecking behaviour which contributed to mortality. Therefore, the NAb isotype titers were only related with health-related survival. I suggest to check for the cause of mortality before the relationships between NAb isotype titers and survival was studied, especially in the non-beak trimmed laying hens. In chapter 2, the logistic regression analysis within breed (White Leghorn and Rhode Island Red, respectively) indicated that the odds ratios for IgM and IgG in brown birds were less than those in white ones, and that line was a significant effect for survival in white birds. This might result from the fact that the lines within the white birds were genetically diverse in mortality. The birds from the present white birds in general showed more fear response and feather pecking behavior which may induce death than birds from the present brown birds (Uitdehaag et al., 2008). Furthermore, the lines within the white birds showed varying levels of feather pecking. The line WF of white birds was characterized as a high-feather-pecking line in earlier experiments (Rodenburg et al., 2003), and line WB was a more gentle line. In this study, the cause of death was not investigated. We speculated that the brown layers may mostly die of individual health-related causes, while white birds layers may die of both health-related causes and social interaction causes, such as feather pecking. That may explain why line was a significant effect for survival in the white birds, but not in the brown layers. This may also explain why isotype titers were more sensitive and accurate parameters for survival in the brown layers (based on the odds ratios). Therefore in chapter 4, the offspring of 25 sires were beak trimmed whereas the offspring of another 25 sires were kept with intact beaks. So that the relationships between NAb isotype titers and survival in beak trimmed and non-beak trimmed laying hens can also be compared to confirm our previous hypothesis that non-health-related causes of mortality (severe feather pecking and cannibalism) may overrule the anticipated relationships between NAb isotype titers and survival in birds with intact beaks.

For the birds used in chapter 2, they were housed in cage system with intact beak (not beak trimmed). A population of the same lines was also housed in cage system, but the laying hens were beak trimmed. For the same purebred laying line, the survival difference between beak trimmed and non-beak trimmed laying hens was generally small (Table 6.2). For most of the lines, survival of beak trimmed is higher than the non-beak trimmed. However, some layer lines, B3, for example, survival of

non-beak trimmed is higher. However, the survival of beak trimmed is significantly different from that of non-beak trimmed crossbred, based on the observation of crossbred female offspring of W1 line and WB line (chapter 4). The beak trimmed hens had high survival of 93.1% and survival days of 447.8 days. The hens with intact beaks had low survival rate of 69.5% and average survival days of 383 days. Therefore, we speculated that part of non-survival caused by feather pecking in purebred laying hens was much less than that in crossbred laying hens. The relationship between NAbs isotype titers and survival detected in chapter 2 was still valid to a large extent.

Table 6.2[1] Survival and survival days in beak trimmed and non-beak trimmed laying hens.

Breed	Line	Beak trimmed			Non-beak trimmed			Survival Difference[2](%)
		n	Survival (%)	Survival days	n	Survival (%)	Survival days	
Rhode Island Red	B1	235	89.8	477	200	86.5	478±58	3.3
	B2	340	93.8	486	200	92.0	484±57	1.8
	B3	144	88.9	486	180	95.6	493±34	-6.7
	BA	488	90.4	484	200	91.0	482±58	-0.6
	BB	266	87.6	478	244	87.3	481±57	-0.3
	BE	385	88.6	483	230	82.2	471±75	6.4
White Leghorn	W1	249	89.6	484	197	86.3	480±57	3.3
	WA	250	97.6	492	210	94.8	490±43	2.8
	WB	340	87.1	479	204	85.8	476±68	1.3
	WC	378	87.0	474	233	81.5	472±72	5.5
	WD	279	92.1	487	206	92.3	485±49	-0.2
	WF	212	92.4	483	200	93.0	484±58	-0.6

[1] Adapted from Star et al. (2007)
[2] Survival difference = survival of beak trimmed – survival of non-beak trimmed

6.3.6. Trade-off between NAb isotype titers with other traits of concern in laying hens

Research exploring the energetic basis for immunity in an evolutionary context has focused on the humoral or cell-mediated branches of the adaptive immune system (Lochmiller and Deernberg 2000). Adaptive immune defenses are costly in terms of energy and nutrient (Lochmiller and Deerenberg 2000). Because of limited nutrient and energy resources, trade-offs are expected between immune system and other

energy-consuming traits like reproduction and body growth (Klasing and Austic 1984b, a; Klasing et al., 1987; Mills et al., 2010). Antibiotics were used for a long time as growth promoters to stimulate growth by keeping the immune system from being activated.

Immune competence could very well be the most important determinant of health and survival for many species. However, as immune systems stimulated for defense channel nutrients away from growth (Soler et al., 2003) and reproduction (Bonneaud et al., 2003), an intermediate immune response in wild animals may often be advantageous (Martin and Coon 2010). At the present stage when the health and welfare problems of the livestock are widely recognized by the breeders who also committed to improve, however, the compromised production efficiency is unwanted, either. In practice, it is necessary to check the trade-off situation between the selection criteria (representive of immunity) and production. Furthermore, as selection criteria, a target selecting value or breeding trend should be clear.

With the overall goal of enhancing animal health, several direct selections for adaptive immune response in poultry were performed for many years (Gross et al. 1980; Pinard et al., 1992; Kean et al., 1994). Correlated responses of production traits were also observed. For example, from White leghorn birds selected for antibody responses to SRBC (Gross et al., 1980), body weight of the high antibody response line at 28 days of age was 10% less than the low line from generation 3. The difference increased to 20% in generation 20. Furthermore, the sexual maturation of the low line birds was 30 days earlier than high line birds (Pinard et al., 1998). This shows the SpAb levels response to a stimulation may negatively affect the growth by taking the nutrient away. A similar selection was performed in a Rhode Island Red laying hens population (Pinard et al., 1992). Body weight at 5 and 17 weeks of age of high antibody line birds from generation 14 was 10% less than the birds from low line (Parmentier et al., 1996). Hens of the low line sexually matured earlier, starting to lay their first egg one week earlier than the high line birds. This result was in line with the finding by Gross et al. (1980) and also support the view that trade-off existed between adaptive immune response and growth and reproduction.

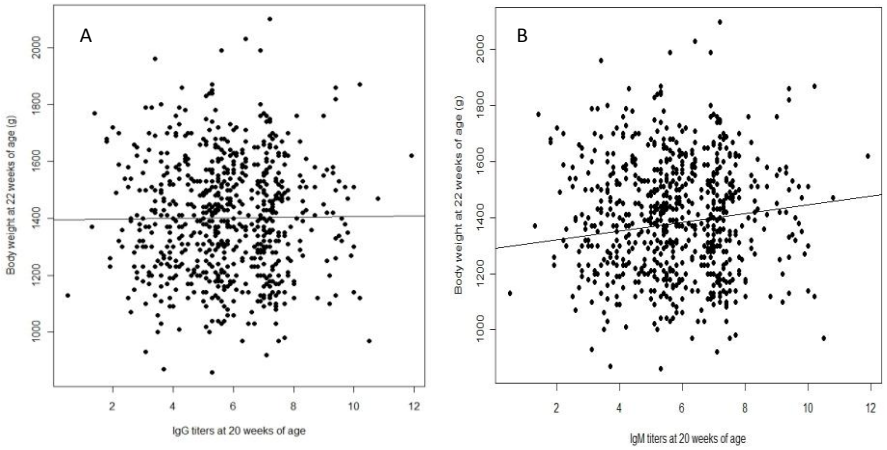

Figure 6.1 Scatter plot illustrating the relationship between average body weight at 22 weeks of age and IgG (A) or IgM (B) titers in serum binding KLH at 20 weeks of age in the 12 purebred layer lines.

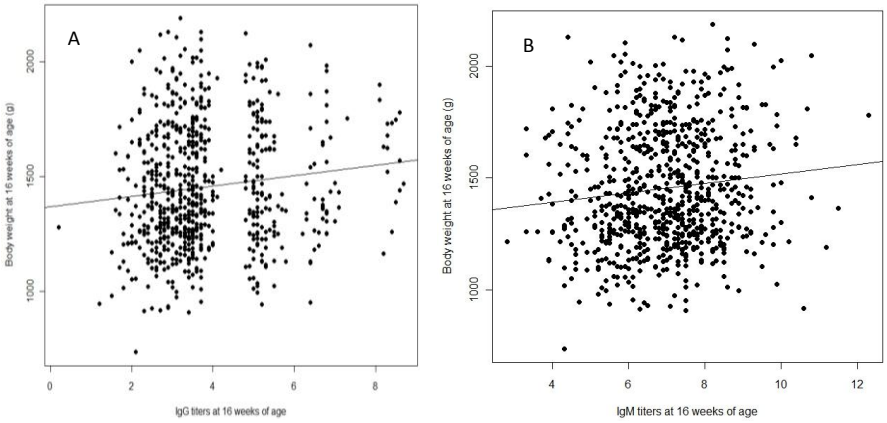

Figure 6.2 Scatter plot the illustrating the relationship between average body weight at 16 weeks of age and IgG (A) or IgM (B) titers in serum binding KLH at 16 weeks of age in the second generation of laying hens which were divergently selected for total NAb titers binding KLH.

However, it is worthy to mention that the real trade-off relationships between NAb and production efficiency might be complex. The birds which maintain a higher level of NAb isotype titers which cost energy and nutrient may show lower production efficiency. On the other hand, these birds may also show higher production efficiency, because the higher level of NAb isotype will protect the animals from initiating the even more costly adaptive immune response by providing first line of defense.

In the study described in chapter 2, all laying hens were housed in cages with 4 chickens of the same line in each cage. It was not possible to monitor the egg-production of each laying hen. We therefore investigated if the higher NAb isotypes levels negatively affected the body growth instead. In contrast to the predicted energetic trade-offs between immune system and body growth, our data showed that the layers with higher levels of IgM or IgG were not accompanied by less body weight at 22 wk of age (Figure 6.1). Since 2009, divergent selection for total NAb titers binding KLH was started. In the second generation, the high NAb line birds showed significantly high body weight than low NAb line birds (Figure 6.2). These results supported the idea that different from the selection for adaptive immune response, the selection for naturally present antibody of innate immunity may be accompanied by larger body size. The less energy or nutrients required for maintain higher NAb isotype level, or the speculation that higher NAb isotype level prevents the costly adaptive immunity from being initiated can be explanations. As yet the trade-off between NAb levels and egg-production still awaits further study.

7.4 Conclusions

In summary, the present and future legislation regarding housing system, welfare, and veterinary treatment in the global laying hen industry encourages the breeders to put more emphasis on the health of the birds in addition to production efficiency. As an important component of innate immunity, NAb may reflect the overall level of defense the laying hens build up genetically for the disease resistance. Especially IgM isotype has shown its protective effect on risk of dying (of health-related reasons) in clinically healthy birds. Diminishing of production efficiency or feather pecking is less likely accompanied with selecting for higher NAb isotype titers. Therefore, NAb, especially IgM isotype titers is a promising trait for future study and implementing into the breeding goal for laying hens for improved health.

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Summary

Summary

Worldwide, especially in Europe, poultry industry is undergoing important changes including ban of the battery housing system and prohibition of beak trimming of the laying hens. The former can facilitate more spread of infectious diseases, and the latter will contribute to higher mortality because of severe feather pecking. Furthermore, given the growing global social concern about food safety and human health, abundant use of antibiotics will either be prohibited or restricted. Furthermore, it is the layer breeding companies' ambition to develop a longer egg production cycle of 100 weeks, during which time the laying hens will be capable of producing 500 eggs without forced-molting process. Breeding goals are the characteristics specified for genetic improvement. These changes or challenges further emphasize the importance of implementing general disease resistance in layers breeding goals next to maintaining high production. The research described in this thesis aimed to (1) find out proper immune parameters which are associated with and predictive for survival of laying hens, and which can be implemented in the breeding program for improved survival of laying hens (chapters 2 and chapter 4), (2) estimate the genetic parameters and reveal the associated genetic regions of the predictive parameters (chapter 3), (3) investigate the relationship between the parameters and feather pecking behavior (chapter 5 and chapter 6).

Natural antibody (NAb), which are the antibodies present in normal healthy animals in the absence of a deliberate antigen exposure are an important humoral part of innate immunity. Previous studies showed that titers of total NAb binding KLH were indicative for a higher probability that chickens survive a laying period. However, only total NAb titers was studied without distinguishing different isotypes. These various functions suggest that NAb isotypes may be differently related to health and survival. In **chapter 2** of this thesis, to identify possible relationships between survival and titers of NAb isotypes in serum of laying hens, birds from 12 purebred layer lines of two commercial breeds, Rhode Island Red (R breed, n = 524) and White Leghorn (W breed, n = 538), were monitored for survival during one laying period (from 20 until 70 weeks of age). Titers of NAb isotypes IgM and IgG binding KLH in serum were measured at 20, 40, and 65 weeks of age, respectively. Overall, the titers of IgM and IgG binding KLH decreased with aging. At the same age, lines within breed showed significantly different titers of isotypes ($P < 0.001$). Multivariable logistic regression analysis showed that NAb isotypes titers at 20 weeks of age were associated to survival of 20 to 40 weeks of age. In the R breed, odds ratios of 0.56 ($P < 0.001$) for IgM and 0.72 ($P = 0.02$) for IgG were

estimated; in the W breed, these were 0.74 ($P < 0.01$) and 0.99 ($P = 0.95$) for IgM and IgG respectively. We conclude that titers of NAb especially the IgM isotype binding KLH at 20 weeks of age are indicative for survival during the laying period. The higher the titers of NAb isotypes, the higher the probability of the layers to survive. Therefore, we showed in chapter 2 that serum levels of natural antibody (NAb) isotypes IgM and IgG binding KLH promising traits for future implementation in breeding programs for higher survival of layers. Another prerequisite for the traits to be implemented into the breeding program is the existence of genetic variation for the traits. Without genetic variations, there is no space for the improvement of the traits by selection. Furthermore, estimation of genetic parameters for the traits of interest is also important for predicting the selection response, setting up correct breeding program and estimating individual breeding value. Therefore in **chapter 3**, we first estimated the genetic parameters for the two isotypes at 20, 40, and 65 weeks of age (IgM20, IgM40, and IgM65; IgG20, IgG40, and IgG65). Pooled genetic parameters were estimated from the total population of 2,504 hens from nine purebred layer lines, with line included in the model to account for admixture. Moderate heritabilities (0.14 - 0.44) indicated that selection for isotype titers is feasible, especially for IgM, which show higher heritabilities than IgG. To better understand the genetic control of NAb isotypes, thus disclosing opportunities to breed for higher survival in laying hens, in the second part of chapter 3, associations between 1,022 single nucleotide polymorphism (SNP) markers and the above-mentioned six immunological traits were estimated in 650 genotyped hens from the nine lines. The association study was performed across lines to detect markers that are closer to the QTL and have the same phase of association in the entire population. Finally, forty-three significant associations between SNPs and isotype titers were detected. The SNPs of interleukins (IL) *IL10* and *IL19* were associated with both isotypes; SNPs of tripartite motif containing 33 (*TRIM33*) and *IL6* showed significant association with IgG20 and IgM20, respectively; SNPs of heat shock protein 90kDa alpha (cytosolic), class B member 1 (*HSP90AB1*) was associated with IgG titers at older ages. Some detected SNPs were also reported associated with other immune and behavioral traits. The majority of commercial laying hens are crossbred. Genetic parameters of NAb isotypes were estimated and relationships between survival and NAb isotypes titers in beak trimmed and non-beak trimmed crossbred laying hens were investigated in **chapter 4**. In total, 1,555 beak trimmed and 1,169 non-beak trimmed crossbred laying hens were used. Genetic parameters of IgM and IgG titers binding KLH at 24 weeks of age were estimated with a linear animal model. The heritabilities of NAb isotypes IgG and IgM were 0.21 (SE = 0.04) and 0.26 (SE =

0.04), respectively. The genetic correlation between IgG and IgM isotypes was 0.43 (SE = 0.11). These results indicated that NAb isotype titers were heritable traits in the crossbred laying hens. Both NAb isotypes can be selected for simultaneously as the detected positive genetic correlation (0.43, SE = 0.11) between them is positive. Furthermore, both row and level of the cage were indicated to be associated environmental factors for NAb isotype titers. Different from an earlier study with purebred hens, survival analysis showed no significant associations of survival with NAb isotype titers in beak trimmed or non-beak trimmed crossbred hens. Non-health-related causes of mortality, especially in birds with intact beaks, overruled the anticipated relationships between NAb isotype titers and survival. The genetic link between feather pecking and NAb isotype titers was indicated in chapter 3. However, more severe feather pecking behavior is not wanted by breeders along with the selection for high NAb titers. Therefore, in **chapter 5**, genetic architecture of laying hens welfare-related traits, feather pecking was analyzed using a traditional linear model and a direct- associative effect model. Furthermore, the relationships between performing/receiving feather pecking and NAb isotype titers were also investigated. The results indicated that individual's NAb isotypes titers did not related with receiving feather pecking, but individual's IgG titers showed a suggestive effect on performing feather pecking to the cage mates which need further confirmation. Finally, in the general discussion described in **chapter 6**, important points of the thesis are highlighted and discussed. It is argued that breeding for general resistance by selecting for innate immunity traits are a good supplementary strategy for improving the survival of the laying hens and prepare the laying hens for the present and future challenges in the industry. The advantages of using NAb isotypes instead of total NAb titers as selection criteria were discussed from the respects of measuring technique, biological meaning of the traits, genetic background. The inconsistence relationships between NAb isotype titers in purebred and crossbred laying hens were mainly attributed to the different cause of non-survival. Future studies about NAb were also suggested.

Overall, the present studies indicate that it is possible to implement NAb especially the IgM isotype titers binding KLH into the breeding goals of laying hens to improve the health-related survival.

S

Samenvatting

Samenvatting

Wereldwijd, maar vooral in Europa, ondergaat de pluimvee-industrie belangrijke veranderingen, waaronder het verbod op het gebruik van het batterij huisvestingssysteem en het verbod op het snavelkappen van leghennen. Het verbod op het gebruik van batterijen kan de verspreiding van besmettelijke ziekten vergemakkelijken, en het verbod op snavelkappen zal bijdragen aan een hogere mortaliteit als gevolg van ernstig verenpikken. Bovendien zal, gezien de groeiende mondiale maatschappelijke bezorgdheid over voedselveiligheid en de gezondheid van de mens, overvloedig gebruik van antibiotica worden verboden of beperkt. De veredelingsbedrijven van legpluimvee hebben de ambitie voor een langere eierproductie cyclus van 100 dagen, gedurende welke tijd de legkippen in staat moeten zijn 500 eieren zonder een geforceerde rui te leggen. Fokdoelen zijn de voor genetische verbetering in aanmerking komende kenmerken. Deze fokdoelen of uitdagingen benadrukken het belang van de algemene weerstand van leghennen naast behoud van een hoge productie. Het in dit proefschrift beschreven onderzoek is gericht op (1) het vinden van goede immuun-parameters die geassocieerd worden met en voorspellend zijn voor overleving van legkippen, en die in het fokprogramma kunnen worden geïmplementeerd voor een betere overleving van legkippen (hoofdstuk 2 en hoofdstuk 4) , (2) een schatting van de genetische parameters, en het identificeren van genetische regio's die gerelateerd zijn met de voorspellende (immuun) parameters (hoofdstuk 3), (3) het onderzoeken van de relatie tussen de immuun parameters en het gedrag van verenpikken (hoofdstuk 5 en hoofdstuk 6).

Natuurlijke antilichamen (NAb) zijn de antilichamen die aanwezig zijn in normale gezonde dieren in afwezigheid van een opzettelijke blootstelling aan antigeen. Zij vormen een belangrijk humoraal deel van het aangeboren immuunsysteem. Eerdere studies toonden aan dat de titers van het totaal gehalte aan NAb dat bindt aan KLH indicatief waren voor een hogere kans dat kippen een legperiode overleven. Echter, alleen de totale NAb titers werden bestudeerd zonder onderscheid te maken tussen de verschillende antilichaam isotypen. De verschillende functies van isotypen suggereren dat NAb isotypen verschillend gerelateerd kunnen zijn aan de gezondheid en overleving. In **hoofdstuk 2** van dit proefschrift, waar mogelijke relaties tussen overleven en titers van NAb isotypen in serum van legkippen werd bestudeerd, werd de overleving bepaald van kippen van 12 zuivere elite lijnen afkomstig van twee oorspronkelijke (commerciële) rassen, Rhode Island Red (R ras, n = 524) en Witte Leghorn (W ras, n = 538) tijdens een

legperiode van 20 tot 70 weken oud. Titers van de NAb isotypen IgM en IgG in serum die bonden aan KLH werden gemeten op 20, 40 en 65 weken leeftijd. Over het algemeen namen de titers van IgM en IgG, die KLH binden, af met het ouder worden. Op dezelfde leeftijd hebben lijnen binnen een ras hebben zeer verschillende titers van de isotypen (P < 0,001). Uit multivariabele logistische regressie analyse bleek dat NAb isotype titers op een leeftijd van 20 weken geassocieerd werden met overleving in de periode van 20 tot 40 weken. In het R ras, werden Odds ratio's van 0,56 (P < 0,001) voor IgM, en 0,72 (P = 0,02) voor IgG, respectievelijk, geschat. In het W ras, waren de Odds ratio's 0,74 (P < 0,01) en 0,99 (P = 0,95) voor IgM en IgG, respectievelijk. We concluderen dat de van de NAb titers vooral het IgM isotype dat bindt aan KLH op 20 weken leeftijd indicatief is voor overleving tijdens de legperiode. Hoe hoger de titers van de NAb isotypen, hoe hoger de waarschijnlijkheid van overleving. Daarom toonden we in hoofdstuk 2 aan dat de serumspiegels van de natuurlijke antilichaam (NAb) isotypen IgM en IgG die KLH binden veelbelovende eigenschappen hebben voor toekomstige implementatie in fokprogramma's gericht op een betere overleving van leghennen. Een andere voorwaarde voor het opnemen van kenmerken in fokprogramma's is het bestaan van genetische variatie. Zonder genetische variatie, is er geen ruimte voor de verbetering van de gekozen eigenschappen via selectie. Bovendien is het schatten van genetische parameters van eigenschappen ook belangrijk voor het voorspellen van de selectierespons, het opzetten van een correct fokprogramma, en schatten van de individuele fokwaarde. Daarom hebben we in **hoofdstuk 3** de genetische parameters geschat voor de twee isotypen op 20, 40 en 65 weken oud (IgM20, IgM40 en IgM65; IgG20, IgG40 en IgG65). Gepoolde genetische parameters werden geschat op basis van de totale populatie van 2.504 kippen uit negen raszuivere leglijnen, met lijn opgenomen in het model om rekening te houden met vermenging. Matige erfelijkheidsgraden (0,14-0,44) gaven aan dat selectie voor isotype titers haalbaar is, vooral voor IgM, die een hogere erfelijkheidsgraad vertoonde dan IgG. Om beter inzicht te krijgen in de genetische controle van NAb isotypen, wat zou bijdragen aan de mogelijkheden om te fokken voor een hogere overlevingskans bij legkippen, werden in het tweede deel van hoofdstuk 3, associaties tussen 1022 single nucleotide polymorfisme (SNP) merkers en de hierboven genoemde zes immunologische eigenschappen geschat in 650 gegenotypeerde kippen uit de negen lijnen. De associatie studie werd over lijnen uitgevoerd om merkers te vinden die dichter bij de Quantitatief Trait Locus (QTL) liggen, en dezelfde fase van associatie hebben in de hele populatie. Uiteindelijk werden drieënveertig significante associaties tussen SNPs en isotype titers gedetecteerd. De SNPs van de interleukines (IL) *IL10* en *IL19* waren geassocieerd

met beide isotypen; SNPs van het *tripartite* motief met daarin 33 (*TRIM33*) en IL6 toonde significante associaties met IgG20 en IgM20, respectievelijk; en SNPs van de heat shock eiwitten 90kDa alpha (cytosol), klasse B lid 1 (*HSP90AB1*) werden geassocieerd met IgG titers op oudere leeftijd. Sommige gedetecteerde SNPs werden ook vermeld vanwege hun verband met andere immuun- en gedragskenmerken. De meerderheid van de commerciële legkippen bestaat uit een kruising. Genetische parameters van NAb isotypen werden geschat, en relaties tussen overleven en NAb isotype titers bij snavel gekapte en niet-gekapte legkippen van een kruising werden in **hoofdstuk 4** onderzocht. In totaal 1,555 gekapte en 1,169 niet-gekapte dieren werden gebruikt. Genetische parameters van IgM-en IgG-titers gericht tegen KLH op 24 weken leeftijd werden geschat met een lineair diermodel. De erfelijkheidsgraad van IgG en IgM was 0,21 (SE = 0,04) en 0,26 (SE = 0,04), respectievelijk. De genetische correlatie tussen IgG en IgM isotypen was 0,43 (SE = 0,11). Deze resultaten gaven aan dat NAb isotype titers ook in een kruising erfelijk zijn. Voor beide NAb isotypen kan gelijktijdig worden geselecteerd vanwege de gedetecteerde positieve genetische correlatie (0,43, SE = 0,11). Verder lijken zowel rij en het niveau van de kooi waarin de dieren waren gehuisvest mogelijke geassocieerde omgevingsfactoren voor NAb isotype titers. Anders dan in een eerdere studie met raszuivere kippen, toonde een survival analyse geen significante associaties aan tussen overleving en NAb isotype titers in gekapte of niet-gekapte kippen. Sterfteoorzaken die niet gerelateerd zijn aan gezondheid hebben, vooral bij vogels met intacte snavels, meer impact dan de verwachte relaties tussen NAb isotype titers en overleving. Het genetische verband tussen verenpikken en NAb isotype titers werd aangeduid in hoofdstuk 3. Echter, ernstig verenpikgedrag dat samen gaat met selectie voor hoge NAb titers is niet gewenst. Daarom is in **hoofdstuk 5** de genetische architectuur van welzijn-gerelateerde kenmerken van legkippen en verenpikken geanalyseerd met behulp van een traditioneel lineair model en een direct-associatief effect model. Bovendien werden ook de relaties tussen de uitvoerende / ontvanger van verenpikken en NAb isotype titers onderzocht. De resultaten gaven aan dat individuele NAb isotype titers niet gerelateerd zijn met het gepikt worden, maar individuele IgG titers suggereerden wel een effect op het uitvoeren van verenpikken op kooigenoten, echter verdere bevestiging is daarvoor nodig. Ten slotte zijn in de algemene discussie **in hoofdstuk 6** belangrijke punten van dit proefschrift gemarkeerd en besproken. Er wordt betoogd dat fokkerij op algemene weerstand door het selecteren op eigenschappen van aangeboren immuniteit een goede aanvullende strategie is ter verbetering van de overleving van leghennen en de voorbereiding van leghennen om te kunnen gaan met de huidige en toekomstige uitdagingen in

de legpluimvee industrie. De voordelen van het meten van NAb isotypen in plaats van het meten van totale NAb titers als selectiecriteria werden besproken vanuit het gezichtspunt van de meettechniek, de biologische betekenis van de kenmerken, en de genetische achtergrond. De inconsistente relaties tussen NAb isotype titers en overleving in raszuivere en gekruiste legkippen werden hoofdzakelijk toegeschreven aan de verschillende oorzaken van sterfte. Toekomstige studies aan NAb werden ook voorgesteld.

Samengevat, de huidige studies tonen aan dat het mogelijk is NAb, vooral de titers van het IgM isotype dat KLH bindt, te implementeren in fokdoelen om de gezondheid gerelateerde overleving van leghennen te verbeteren.

P

Publications

Peer-reviewed publications

Y. Sun, E. D. Ellen, J. J. van der Poel, H. K. Parmentier, P. Bijma, 2013. Modelling of feather pecking behaviour in beak trimmed and non-beak trimmed crossbred laying Hens: variance component and trait-based approach. Submitted to Poultry Science.

Y. Sun, E.D. Ellen, H. K. Parmentier, J. J. van der Poel. 2013. Genetic parameters of natural antibody isotypes and survival analysis in beak trimmed and non-beak trimmed crossbred laying hens. Poultry Science, 92(8): 2024-2033.

Y. Sun, F. Biscarini, H. Bovenhuis, H. K. Parmentier, J. J. van der Poel. 2013. Genetic parameters and across-line SNP associations differ for natural antibody isotypes IgM and IgG in laying hens. Animal Genetics, 44(4): 413-424.

Y. Sun, H. K. Parmentier, K. Frankena, J. J. van der Poel. 2011. Natural antibody isotypes as predictors of survival in laying hens. Poultry Science, 90(10): 2263-2274.

H. L. Zhou, L. H. Gu, Y. Sun, T. S. Xu, G. Rong, W. L. Xia. 2013. Genetic and phenotypic parameter estimates for growth traits of Hainan black goat in south china. Submitted to Animal Production Science.

T. S. Xu, L. H. Gu, Y. Sun, M. Xie, X. H. Zhang, B. G. Ye, X. L. Liu, S. S. Hou, 2013. Characterization of MUSTN1 gene and its relationship with skeletal muscle development at postnatal stages in Pekin ducks. Submitted to Genetics and Molecular Research.

L. Meng, R. X. Jia, Y. Sun, Y. J. Wan, Y. L. Zhang, Z. Y. Wang, B. S. Zhong, G. M. Zhang, X.Q. Wang, F. Wang, 2013. Growth Regulating, Imprinting, and Epigenetic Transcription-related Genes Expressions differ in the Lung of Deceased Transgenic Cloned and Normal Goats. Submitted to Theriogenology.

L. Meng, Y. J. Wan, Y. Sun, Y. L. Zhang, Z. Y. Wang, Y. Song, F. Wang, 2013. Generation of Five Human Lactoferrin Transgenic Cloned Goats Using Fibroblast Cells and Their Methylation Status of Putative Differential Methylation Regions of IGF2R and H19 Imprinted Genes. Submitted to PloS one.

A

About the author

Yanyan Sun (孙研研) was born on the 10th of Dec 1984 in Jiangsu province, P. R. China. She obtained her high school diploma in 2007 from No. 1 high school of Xinyi. In autumn of the same year, she started her bachelor study in China Agricultural University (CAU) in Beijing, specialized in Animal Science. The subject of her thesis for the bachelor degree was "The application of Microsatellite Technology in identifying the genetic relationships among different chicken flocks". After getting the bachelor diploma in 2007, she continued her Msc study in Animal Breeding and Genetics in CAU and got the Msc diploma in 2009. The subject of her Msc study was "Gene silencing of *Myostatin* by small interfering RNA in broiler". She was granted by the China Scholarship Council and started her Ph.D. in the Animal Breeding and Genomics Centre in Wageningen University since September of 2009. She worked on the project entitled "Immunogenetic analysis of natural antibody isotypes in laying hens" which resulted in this thesis. Simultaneously, she joined Chinese Association of Life-sciences in the Netherlands and European Chinese Biomedical and Biopharmaceutical Federation, and actively worked as a member of the council. Since November 2013, she is employed as a junior researcher at the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences.

Training and Education

Training and Education

The Basic Package (3.0 ECTS)	year
WIAS Introduction Course	2010
Ethics and Philosophy in Life Sciences	2011

Scientific Exposure (12.2 ECTS)	year
International conferences	
NE-1034: Genetic Bases for Resistance and Immunity to Avian Disease, Ithaca, US	2011
12th Avian Immunology Research Group meeting, Edinburgh, UK	2012
NE-1034: Genetic Bases for Resistance and Immunity to Avian Disease, East lansing, US	2012
Seminars and workshops	
WIAS Science Day	2010-2013
How to write a world-class paper, Wageningen, the Netherlands	2011
15th QTL-MAS Workshop, Rennes, France	2011
5th Combined Workshop Fundamental Physiology and Perinatal Development in Poultry, Wageningen, the Netherlands	2011
Hendrix Genetics Academy: Connecting Science & Industry, Boxmeer, the Netherlands	2012
Genetics of social life: Agriculture meets evolutionary biology, Wageningen, the Netherlands	2013
Presentations	
NE-1034: Genetic Bases for Resistance and Immunity to Avian Disease, Ithaca, US (oral)	2011
WIAS Science day, Wageningen, the Netherlands (poster)	2011
12th Avian Immunology Research Group meeting, Edinburgh, UK (poster)	2012
NE-1034: Genetic Bases for Resistance and Immunity to Avian Disease, East lansing, US (oral)	2012
WIAS Science day, Wageningen, the Netherlands (poster)	2013

In-Depth Studies (6.4 ECTS)	year
Disciplinary and interdisciplinary courses	
Genomic Selection in Livestock, Wageningen, the Netherlands	2011

Social genetic effects: Theory and genetic analysis, Wageningen, the Netherlands	2013
Advanced statistics courses	
Design of Experiment	2010
Statistic for Life Sciences	2010
PhD students' discussion groups	
ABG PhD Paper Discussion Group	2012-2013

Professional Skills Support Courses (4.3 ECTS)	year
Research Master Cluster	2009
Information Literacy including EndNote Introduction	2010
Project and Time Management	2011
Techniques for Writing and Presenting a Scientific Paper	2011

Research Skills Training (6.9 ECTS)	year
Preparing own PhD research proposal	2009
Introduction to R for Statistical analysis	2010
Getting started with ASReml	2011

Statutory Courses (3.0 ECTS)	
Use of Laboratory Animals (mandatory when working with animals)	2010

Training and education total	**35.8 ECTS**

A

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Wageningen, The Netherlands

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