Different genetic bases of immune responses in laying hens.

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Different genetic bases of immune responses in laying hens

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Abstract: Given the growing concerns of consumers regarding the safety of animal-derived foods, it is important to develop effective methods to reduce chronic use of antibiotics and to enhance the effectiveness of vaccine protection by improving, via genetic selection, the innate ability of birds to respond to antigenic challenges. Immune responses to different pathogens follow distinct pathways and resistance to most diseases and pathogens is controlled by polygenes. Therefore the aim of the work described in this thesis is to identify genetic factors underlying immune response in laying hens. The H/L population used in this study, originate from a cross of two lines divergently selected for high (H) and low (L) primary antibody response to Sheep Red Blood Cells (SRBC). Individuals from generations F0, F1 and F2 were genotyped with 180 microsatellite markers. The phenotypes were collected for entire F2 population with regard to several antigens: SRBC, KLH, E.coli, *M.butyricum*.

The setup of the experiment allowed the use of two statistical models to analyze the data: a paternal half-sib model and a line-cross analysis model. In the half-sib analysis, QTL for the SRBC primary antibody response were identified on GGA1 and on GGA16. In the line-cross analysis Mendelian QTL were identified: on GGA4, GGA7, and GGA13. Subsequently, QTL analysis was performed on the data for two other antigens: KLH and *M.butyricum.* These analyses were performed on two independent populations: H/L cross and FP (feather pecking) lines. A QTL for the primary antibody response to KLH detected on GGA14 was validated in both populations under the half-sib analysis model. Based on the results differences in the genetic regulation of antibody responses to two different T-cell dependent antigens, KLH and *M. butyricum* were suggested.

Subsequently the question asked was: which genetic components of innate immune responses are identified and which relations between the innate and the adaptive immune system could be found. Natural antibodies in our studies were measured by level of antibodies binding the homotopes LPS and LTA in the two, already mentioned populations: H/L and FP cross. For the H/L population data were collected at two ages: 5 wks of age and 18 wks of age, whereas for the FP cross data were collected at 37 wks of age. The QTL results indicate that different regions are associated with levels of antibodies binding the homotopes LPS and LTA, respectively, in poultry and that different genetic regions are involved in innate (non specific) and adaptive (specific) immune responses.

The QTL region detected for primary antibody response to KLH on GGA14 was fine mapped. Previously collected genotypic data was combined with additional information obtained by genotyping individuals with SNP (Single Nucleotide Polymorphism) markers. A combined linkage disequilibrium / linkage analysis (LDLA) was performed. This analysis resulted in narrowing down QTL region from initial 50cM to the region below 1cM.

Given the strong immuno-modulating features of KLH, and the high level of Nabs towards KLH in (non immunized) poultry we propose to focus on the QTL on GGA14 by future studies.

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.Chicken immunity

1.1. Innate immunity

The immune system might be defined as a weapon of mass destruction evolved during evolution to protect living organisms. All living organisms have protective mechanisms, e.g., bacteria have restriction enzymes, insects have toxic and lytic agents, and plants have degrading enzymes. In vertebrates both "innate" and "adaptive" biodestructive mechanisms can be distinguished (Cohn M. 2002). For a long time the potential of innate immune system has been under estimated. Innate immunity is represented by natural barriers as skin, physiological factors such as: pH, temperature, oxygen tension, lysozyme, and phagocytic cells. Innate immunity involves also cells as natural killer cells (NK), mast cells, dendritic cells and phagocytes. Phagocytes, which might be subdivided into two classes: neutrophils and macrophages, both engulf microbes. NK cells role is to attack virally – infected cells. NK cells release perforins and granzymes from its granules, which induces apoptosis. NK cells secrete Interferon-γ to prevent healthy host cells from becoming infected but also to augment the T cell response. Dendritic cells form an important bridge between innate and adaptive immunity, as these cells present antigenic peptides to T helper cells. The innate immunity itself usually terminates infections before the onset of disease. The Toll like receptors (TLR) play an important role in the first line of defense by discriminating between different types of outside threats (recognition of antigens from virus vs. bacteria). The TLR recognition primes the adaptive immune response to react appropriately. (O'Neill 2004).

An important part of the innate immune system is formed by natural antibodies (NAb). They are present in non-immunised individuals. NAb have low binding affinity and a broad specificity repertoire (Casali et al., 1989; Dacie J.V., 1950). In mammals, NAb are mainly of IgM isotype (Boes M., 2000), however also IgA and IgG have been reported (Ochsenbein A.F. et. al., 1999). NAb are probably involved in early recognition and clearance of foreign material. They enhance processes of antigen uptake and antigen presentation via dendrites or B cells. Specific immunity and protection may be also enhanced by NAb and idiotype – anti-idiotype networks (Ochsenbein A.F. et. al, 1999; Tomer Y., et al. 1988). It is very likely that NAb are present in chickens (Jalkanen G et. al. 1983). To date, chicken antibodies binding ovarian antigens (Barua A. and Yoshimura Y., 2001) and MHC class IV were reported (Longenecker B.M. and Mosmann T.R. 1980; Neu N. et al., 1984).

1.2. Adaptive immunity

The vertebrates possess not only innate immunity but also are able to mount defense mechanisms that constitute adaptive immunity. The end point of adaptive immunity is memory, and cells mediating the specific defense: lymphocytes B (B cells) and lymphocytes T (T cells). There are two types of B cell: plasma B cell which secrete antibodies and through them effect the destruction of antigens, and memory B cell which are formed specific to each antigen encountered after the primary immune response. The memory B cells are stored in the circulating system for later use, for a long time possibly a whole lifetime. Among several types of T cell, two principal types are: cytotoxic T cells and helper T cells. Cytotoxic T cells destroy infected cells, whereas helper T cells activate other type of cells in the immune system. Each B cell and T cell is specific for a particular antigen, which means that each cell is able to bind to a particular molecular structure. The specificity of binding resides in a receptor for an antigen: the B cell receptor (BCR) and the T cell receptor (TCR) respectively. The T-cell receptor binds to an antigen presented by another cell called antigen-presenting cell (APC) and, if additional activation signals are present, respond to that antigen. The B cell receptor binds to an antigen which is presented by other antigen presenting cells, T cell receptors and in the end the B cell responds by secreting a large number of soluble forms of receptor called antibodies.

1.3. Antibodies

Antibodies are glycoproteins build of sub-units containing two identical light chains (200 amino acids each) and two identical heavy chains. Both the heavy and light chain have a variable region (100 amino acids in the N terminal end), which vary greatly from antibody to antibody, and a constant region (C). Different kinds of constant regions for both light and heavy chains give different types of antibodies, e.g., IgG, IgA, IgM, and IgD. Variable regions (V) are responsible for epitope recognition, whereas constant regions (C) are responsible for effector functions e.g. binding, agglutination. Genes coding the Ig light chain consist of: variable region genes (V_L), joining region genes (J_L) and constant region genes (C_L) and, are separated by a non-coding DNA region. In man and mouse the non-coding genes are spliced out to allow gene rearrangement and join the V_LJ_L regions in the genomic DNA, and subsequently excision of non-coding region in the RNA transcript what allows the $V_LJ_LC_L$ regions to combine and translate into a functional Ig light chain. In the case of Ig

heavy chain genes one more region is involved: diversity (D) genes, arranging V_HDJ_H in the first place, otherwise the rearrangement process is similar to the L chain rearrangement. The cluster of genes encoding the Ig light chain has only one copy of functional V_L and J_L genes in mature B cells. Similar to the Ig heavy chain, only a single copy of functional V_H and J_H exist. However there are 16D genes between V_H and J_H, the sequence of these genes is very similar. In mammals, the variability of antibodies is generated in a process named "gene conversion". During this process different sets of V-J-(D)-C are combined by splicing and rearrangement. In chicken (bovine and pig) the situation is different. In both the heavy and light chain Ig loci there are clusters of pseudogenes upstream of heavy V and light V genes. There are 80 pseudogenes upstream of V_H loci and 25 pseudogenes in case of V_L genes (Reynard et al., 1987). Only after VJ conversion takes place, and both V_L and V_H genes are replaced by their pseudogenes counterparts, only then a functional gene product is formed.

Following the first exposure to an antigen, activated B cells differentiate into plasma cells. The amount of antibody produced is usually relatively low. Over time, antibody levels decline to the point where they are undetectable. The first type of antibody produced is mainly IgM (although small amounts of IgG are usually also produced). A second injection of antigen produces a secondary response. This secondary response is more specific. During this phase the amount of antibody produced rises to high levels. Antibody levels tend to remain high and for a much longer period of time. The main type of antibody produced is IgG (although small amounts of IgM are sometimes produced). In addition there is a shift from idiotypes with low affinity to idiotypes with higher affinity for the antigen. This process which is based on competition between clones is an important feature of a maturating immune response.

2. Major Histocompatibility Complex

A crucial role in control of the immune response is played by the histocompatibility molecules. Histocompatibility molecules are glycoproteins expressed at the surface of almost all vertebrate cells. Their role is to display antigens in such a way that they can be recognized by T lymphocytes. The T Cell Receptor (TCR) identifies an epitope that is a mosaic of the 14 – 19 amino acid long peptide in the groove and portions of the alpha helices flanking it. The genes encoding the different heavy chains of the MHC are clustered on chromosome 16 in the major histocompatibility complex (MHC). The chicken MHC also

known as B-locus represents the minimal essential major histocompatibility complex. It consists of a 92-kilobase region that encodes only 19 genes, making it approximately 20-fold smaller than human. All the genes have their counterparts in the human MHC, defining the minimal essential set of genes conserved over 200 million years of divergence between birds and mammals (Kaufman et al., 1999). The MHC in chicken encodes the following gene products: class I (BF) - expressed on almost all cell types, class II (BL) - expressed on B cells, APC and stimulated T cells; and classes IV (BG) which is unique to avian expressed mainly on erythrocytes. A lot of research has been focused on the B-complex. The B-complex has been associated with disease resistance. A remarkable feature is association between MHC haplotype and for instance Marek Disease Virus resistance. The most resistant haplotype being B^{21}, the most susceptible being B^{19} ,B^{14}, B^{5} whereas B^{2}, B^{6}, B^{4} fall in between as moderately resistant.

Next to the MHC complex, chicken possess also the pseudo MHC complex called *Rfp-Y*, located on the same microchromosome as the classical MHC. *Rfp-Y* contains a set of class I and class II β genes that segregate independently from the MHC. It was suggested by Miller et al. (1996) that genes within the *Rfp-Y* region differ in function from their counterparts within the B system. The *Rfp-Y* genes, including the c-type lectin gene located in *Rfp-Y*, may represent an alternative form of the MHC, however the functional properties with regard to immune function are still under investigation.

3. Cytokines and Chemokines.

Beside the already mentioned B cells, T cells and histocompatibility molecules, there is a group of soluble mediators called cytokines and chemokines and their receptors that mediate communication between all actors of immune system. Cytokines cover a broad group of molecules, which include interferon, interleukin and chemokine. Cytokines are small proteins or glycoproteins messenger molecules transporting information among cells. Cytokines, together with their receptors and play a role as central regulators of immune system by affecting the activity of other cells (Callard et al., 1999; Davison, 2003). Most of the cytokines are secreted but some of them can also be expressed on the surface of the cell. Many cytokines are produced by more than once cell type and most of them have multiple and diverse biological function. Among the cytokines one can identify: interleukins, growth factors, chemokines and interferons. More than 200 cytokines have been identified in

mammals, most are present in chicken as well, and however, to date not all of them are known, either as gene or in biological function. Nevertheless, from the historical perspective chicken play a special role in the discovery of cytokines. The first interferon (IFN) of chicken was described as an unknown soluble factor interfering with the growth of influenza A virus by Isaacs and Lindenmann (1957). In mammals, there are two major type of IFN (type I and type II) and several families within type I interferon (IFN -α, IFN-β, IFN-ω, IFN-τ) and one type II interferon IFN-γ. Cytokines like IFN-γ are produced by a subset of CD4+ T helper cells. In mammals it is clear that there are two subset of T helper cells: Th1 and Th2. Th1 T cells secrete IL2, TNFα and IFN-γ and are involved in cell mediated immune response. Whereas Th2 T cells secrete IL-4, IL-5, IL-10 and IL-13 cytokines required for antibody production. In poultry, though in general most cytokines were identified, the Th1/Th2 paradigm has not been established; however, Sijben et al. (2001) found differences in antibody response to Th1-like and Th2-like antigens with regard to magnitude, kinetics and modulation of the antibody response. On the other hand, chickens lack some Th2 response components compared to mammals. Chickens have no eosinophils, mast cells and basophils. There are no IgG sub classes and poultry lacks of IgE. In comparison with mammals there are five cytokine genes in the Th2 cluster (IL-3, IL-4, IL-13, GM-CSF) in chicken.

In relation to the immune response, some of the major genes are already mapped on GGA1 (γ-interferon; Guttenbach et al., 2000), GGA15 (immunoglobulin light chain; Zhao et al., 2000), GGA16 (B-region; Dominguez-Steglich et al., 1991), GGA27 (T cell receptor α chain; Wang et al., 1997), GGA4 (IL-2 and IL-8; Reboul et al., 1999).

However with regard to the complexity and diversity of immune responses and disease resistance still a big effort has to be done. Still many genes known to be involved in control of immune response are not described yet in chicken.

4. Chicken genomic resources.

The chicken has been studied extensively because of its economical importance for the poultry industry as well as its biological importance as an animal model for, e.g., growth, development and immunology (Brown et al., 2003).

The haploid chicken genome contains 1.2 x 10^9bp. The karyotype is divided among 8 pairs of large chromosomes, 2 sex chromosomes and 30 pairs of mini – chromosomes (Schmid et

al., 2000). The female is the heterogametic sex (ZW) and the male is homogametic (ZZ). Three reference populations have been used as mapping populations for genetic markers in chicken: the Compton (Bumstead and Palyga, 1992), the East Lansing (Crittenden et al., 1993), and the Wageningen resource populations (Groenen et al., 2000). Based on these resource populations, a consensus genetic linkage maps was build using different types of genetic markers e.g. restriction fragment length polymorphism (RFLP), microsatellites (tandem repeats of mono-, di-, tri- or tetranucleotides), amplified fragment length polymorphism (AFLP) and recently single nucleotide polymorphism (SNP). The consensus linkage map consists of approximately 1,900 markers and is 3,800 centi Morgan in length (Groenen et al., 1998). In recent years a growing number of resources has become available: high-density linkage map (Schmid et al., 2000) BAC library (Crooijmans et al., 2000), RH mapping (Morrison, et al., 2002), multiple cDNA libraries providing large numbers of ESTs (UK website). All these tools enable the application of comparative genetic approaches.

4.1. Quantitative Trait Loci.

Quantitative traits are phenotypes that exhibit quantitative variation. Therefore, studying quantitative traits depends on measuring rather than counting. Genes or loci with an effect on a quantitative trait are called Quantitative Trait Loci (QTL). One or many QTL may influence a trait. In principle the QTL mapping is based on strains, which differ in: 1) alleles affecting the trait of interest and 2) polymorphic molecular DNA markers. Such strains are reproduced to create a F1 cross and followed up by either backcross or an F2 generation, which can be used as a mapping population. QTL mapping involves analysing the genome with one marker at the time. All individuals are divided into marker genotype classes and a statistical test is performed to determine if there is a significant difference in phenotype between the marker genotype classes. In case of such a difference, one assumes that the QTL is linked to the DNA marker. A common design of QTL mapping experiments is to use half-sib families having a large number of offspring. QTL can be assigned to a chromosomal region using linkage analysis. Linkage mapping is based on an effect within a family between the alleles at a marker locus and the alleles at a QTL. When using a genome scan approach, genetic markers covering all chromosomes are used so that a QTL anywhere in the genome can be detected. The most common type of analysis is a half –sib analysis (i.e. paternal half – sib analysis) where, one assumes the sire of the family to be heterozygous at the QTL. In general the power of QTL detection depends on: 1. the

probability of a sire being heterozygous at the QTL locus, this probability increases if it concerns a cross between two distantly related lines or breeds; 2. the magnitude of the QTL effect, 3. Number of individuals per sire analysed in an experiment, since individual QTL effects are usually very small (van Arendonk and Bovenhuis, 2003).

Once detected in a whole genome scan a QTL, should be validated to be sure that the detected effect is not an artefact. The validation should be a study independent from the original QTL experiment. One of the possibilities is to use other related animals through sire or dam lines. Another possibility is to use unrelated animals, e.g., by taking animals from another population. (Bovenhuis &, Spelman 2000).

Subsequently after QTL detection and preferably confirmation in an independent study, the next step is fine mapping. The idea behind fine mapping is to increase genetic information in the linked region by genotyping additional markers and repeat the linkage analysis. The error in the estimation of QTL location may be affected by the amount of the genetic information, the number of families, the family size and the proportion of variation explained by the QTL locus (Atwood and Costa 2003). Fine mapping to a region below 1cM is difficult to achieve and requires more recombination events.

Once the QTL region is narrowed down, it is of interest to find genes underlying the trait of interest. Successful result, however, depends on the size of the critical region and on whether the region is 'gene-rich' or 'gene-poor'. At a certain moment, analyses arrive at the point where the chromosomal region of interest is small enough and all the genes are known. Loci associated with the QTL, might be determined using a strategy based on linkage disequilibrium (LD). The assumption being that haplotypes with identical marker alleles, surrounding the QTL are expected to have similar haplotype effect. Identical markers indicate that the region is identity-by-descent (IBD), and therefore is expected to carry the same QTL alleles. Fine mapping by analysing haplotype effects was successfully applied in the detection of IGF2 (Nezer et al., 1999; Nezer et al., 2003; Van Laere et al., 2003) as a gene related with the muscle growth in pigs and in diary cattle detecting a haplotype associated with increased fat percentage in milk (Riquet et al., 1999).

4.2. Selection Lines.

To create divergent lines, a model that was first developed in Biozzi mice, has been applied in current experiment. In brief, Biozzi et al. modified the immune system of mice by

divergent selection for antibody responsiveness. The decrease in the low line was faster than the increase in the high line. After 18 generations the selection plateau was reached and the mice were considered homozygous for all loci determining trait under selection. There are a number of modifications in the immune system caused by the selection. The number of B lymphocytes was higher in H line (Biozzi et al., 1972; Unanue et al., 1974); catabolism of antigens occurred faster in L line (Biozzi et al., 1975), presentation of antigen was prolonged in the H line (Wiener and Bandieri, 1974). The result of the selection was a 200 fold interline difference between the H and L line for antibody levels and lower threshold dose for T-cell dependent and T-cell independent antigens (Biozzi et al., 1975).

Individuals which have been genetically selected for high production efficiency seem to be more at risk for behavioural, physiological and immunological problems. (Rauw et al. 1998). Selection for increased growth in poultry may be accompanied by decreased specific immune response and disease resistance (Rauw et al., 1998), while selection of several chicken lines for increased immune response resulted in decreased BW (Siegel and Gross, 1980; van der Zijpp 1983). Chickens lines divergently selected for antibody response to SRBC revealed a negative correlation between BW and antibody titers to SRBC (Parmentier et al. 1996). Also selection lines for antibody response to Newcastle disease virus, phytohemagglutinin responses, and phagocytic activity showed negative correlation between antibody titers and BW (Pinard van der Laan, 2002).

4.6. Lines used in the experiment.

The chicken lines, used in the presented experiments, originate from a cross (ISA Warren) between two divergently selected lines for either high (H line) or low (L line) primary antibody responses to sheep red blood cells (SRBC). Selection in the founder lines was based on the individual total antibody titre at 5 days after primary intramuscular immunisation with SRBC at 37 days of age (Van der Zijpp and Nieuwland, 1986). In generation 10, H and L line varied in various immune characteristics. H line had higher response to T cell dependent antigens: Keyhole Lymphet Haemocyanin, TNP, Bovine Serum Albumin (Parmentier et al., 1994). There was no difference between H and L line in the response to T cell independent antigens: *Brucella abortus*, *Salmonella* as well as in macrophage activity (Parmentier et al., 1998). T cell activity in vitro was higher in the L line than in H line. H line was more disease resistance to Marek challenge and Coccidiosis (*Eimeria acervulina* challenge) (Parmentier et al., 2001). H and L line were also different for

the MHC haplotypes. The H line haplotype was B-21 predominantly, whereas the L line haplotype was mainly B-14. The genetic difference between the high (H) and low (L) lines reached 5.1 phenotypic standard deviations in the 18th generation (Bovenhuis et al., 2002). In the same analysis, the heritability for Ab-titer was found to be 0.18 for male and 0.19 for female (Bovenhuis et al. 2002). From the 17th generation 15 males from the L line and 15 females from the H line; and 16 males from the H line and 16 females from the L line were selected to produce reciprocal crosses. Six randomly chosen F_1 males were mated to 12 randomly chosen F_1 dams to produce F_2 birds. Mating of males and females was such that inbreeding was avoided. Six hundred seventy two F_2 individuals were obtained, with an average of 56 offspring per full - sib family and 112 offspring per half – sib family.

5. Disease resistance.

Prevention of outbreaks of infection diseases in poultry may be facilitated by genetic improvement of innate and/or specific resistance to infectious diseases factors. The genetic make up defines the maximum performance of individuals, however, little is known of the genetic basis, which underlay innate and adaptive immune resistance in poultry. It has been suggested that intensive selection for production traits impaired the capability to generate protective immune response and disease resistance. The MHC gene family has been extensively studied for its role in disease resistance and a wide array of pathogens has been demonstrated to be associated with major histocompatibility complex variability but only in part. An important observation from the MHC and disease studies is that, there is not a single haplotype which responds optimally in all genetic backgrounds. The resistance to most diseases is likely controlled by polygenes. Divergent genetic selection for disease resistance can produce genetic lines, which are used to investigate correlated changes in genetics (Lamont, 1998). Siegel and Gross investigated genetic control of humoral immune (SRBC) response in laying hens and tested the resistance of selective lines to infectious diseases. The high–antibody production line was more resistant, to parasites and viruses but not to bacteria when compared with the low antibody production line. The high /low lines, founders of the F2 crossed used for current study, were also investigated for disease resistance to Marek's disesase, interaction and correlation of primary and secondary immune responses. Selection of egg-type chickens to antibody response to Salmonella pullorum antigen was successful (Pevzner et al., 1981). Also meat-type chicken lines were

successfully selected for high and low antibody responses (Pitcovski et al., 1987; Yonash et al., 1996). To be able to apply antibody selection in a program of genetic selection for disease resistance the variation in antibody levels must be associated with variation in response to disease. Fortunately there is evidence for association of antibody level and disease resistance.

Aim and outline of the thesis

As presented in this introduction, the immune system is very complex. Immune responses follow different pathways and resistance to most diseases and pathogens is controlled by polygenes. The first genes studied as markers of immunity were the MHC genes; however, MHC genotypes explained only 3.5% of the total variation. Therefore the aim of the work described in this thesis is to identify genetic factors underlying immune response in laying hens relevant to Antibody response. In chapter 2, a QTL study is described for primary and secondary antibody response to SRBC, which was the selection criterion for the founder lines of the F2 cross used in the current experiment. Different antigens may be influenced by genetic factors, therefore, a QTL study on the primary antibody response to KLH and *M.butyricum* was performed (chapter 3). Although the adaptive immune response is mediated by memory B cells and T cells, the first defence line is created by an innate immune response. Characteristics of innate immune (non-specific) responses, relations between specific and non-specific immune responses, and QTL detected for the innate immune responses were examined (chapter 4). In order to dissect the genetic basis underlying traits of interest, a detected QTL should be validated in an independent population and subsequently fine mapped to narrow down the QTL. This enables the detection of positional candidate genes. This line of investigation was followed for the QTL detected for primary antibody response to KLH on GGA14. This QTL was detected in the H/L population and validated in an independent population (Chapter 2). The fine mapping and haplotype reconstruction approach is described in Chapter 5 of this thesis. In chapter 6 the results of the previous chapters are discussed.

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CHAPTER 2

Detection of QTL for immune response to Sheep Red Blood Cells in laying hens

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Summary

The aim of this study is to detect QTL involved in the regulation of the primary and the secondary immune response to SRBC in a resource population using microsatellite DNA markers. The F2 resource population originates from a cross of two divergently selected lines for either high (H line) or low (L line) primary antibody response to sheep red blood cells (SRBC). The F2 population consisted of 6 half-sib families, 3 families per reciprocal cross. Total antibody titres to SRBC were determined by agglutination in serum from all birds. F0, F1 and F2 generations were genotyped for 170 microsatellite markers, using a whole-genome scan approach. The half-sib and the line-cross analyses were performed to determine QTL regions associated with regulation of the immune response. In the half-sib analysis, two suggestive QTL for SRBC primary response were identified, first QTL on GGA1 second QTL on GGA16. No QTL was identified for SRBC secondary response under the half-sib model. In the line-cross analysis, seven QTL were identified. Three mendelian QTL were detected: on GGA4, GGA7, and GGA13. Four QTL with parent – of – origin effect were identified: on GGA2, GGA3, GGA27 and linkage group E60E04W23 for SRBC primary response A suggestive QTL was identified on GGA18 for SRBC secondary response.

Keywords: chicken, selection, QTL, immune response, sheep red blood cells.

Introduction

Infectious diseases are responsible for the major economic losses in livestock production. Although control of the environment by sanitation and isolation and the use of vaccination and drugs reduce the incidence of many diseases, the problem has not been eliminated (Heller et al. 1992). Given the growing concerns of consumers regarding the safety of animal-derived foods, it is important to develop effective methods to reduce chronic use of antibiotics and to enhance the effectiveness of vaccine protection by improving, via genetic selection, the innate ability of birds to respond to antigenic challenges. There is a wide difference in the myriad of pathogenic organisms to which poultry may be exposed during their life time, and it is difficult to identify few infectious organisms that would serve as the appropriate selection targets (Lamont 1998). The ability of birds or animals to respond to antigenic challenges may be improved indirectly, by selecting for an immune response to non-infectious complex antigens such as sheep red blood cells (SRBC). Chickens selected for high antibody responses to SRBC showed higher resistance to some infectious diseases e.g. *Marek's* and *Newcastle Disease Virus* (Pinard et al. 1992) and *coccidiosis* (Parmentier et al. 2001).

Immune response in chicken, as a complex trait, is affected by quantitative trait loci (QTL) as well as the environment (Gavora 1993). Previous QTL experiments in poultry dealing with immune traits include studies: *Marek Disease Virus* (Vallejo et al. 1998) and antibody response to *Salmonella enteritidis* vaccine (Kaiser et al. 2002), antibody response to *E.coli* vaccine (Yonash et al. 2001). Still little is known of the genetic basis of innate and acquired disease resistance. To create divergent populations, we applied a model successfully used in mice by Biozzi et al. (1979) and based on divergent selection for either high (H line) or low (L line) antibody response to SRBC. The genetic difference between the high (H) and low (L) lines reached 5.1 phenotypic standard deviations in the 18^{th} generation (Bovenhuis et al. 2002). In the same experiment, the heritability for Ab-titer was found to be 0.18 for male and 0.19 for female (Bovenhuis et al. 2002).

We present the results of a study to identify of QTL involved in the regulation of primary and secondary immune responses to SRBC. To our knowledge, we report an unique QTL mapping experiment in terms of phenotypic observation, large number of F2 offspring and number of microsatellite markers used, and a full scale analysis using two genetic models: half-sib and line-cross.

Material and methods

Experimental design.

The F2 population originates from a cross (ISA Warren) between two divergently selected lines for either high (H line) or low (L line) primary antibody response to sheep red blood cells (SRBC). Selection was based on the individual total antibody titre at 5 days after primary intramuscular immunisation with SRBC at 37 days of age (Van der Zijpp and Nieuwland, 1986). From the 17^{th} generation 15 males from the L line and 15 females from the H line; and 16 males from the H line and 16 females from the L line were selected to produce reciprocal crosses. Six randomly chosen F1 males were mated to 12 randomly chosen F1 dams to produce F2 birds. Mating of males and females was such that inbreeding was avoided. Six hundred seventy two F2 individuals were obtained, with an average of 56 offspring per full - sib family and 112 offspring per half – sib family. To create sufficiently large families, eggs were collected during two-week period and then placed in incubators to hatch. In total 6 hatches were made to create the whole F2 population. Hens and cocks were housed in rearing cages with free access to feed and water. The Ethical Committee on Animal Care and Welfare of Wageningen University, The Netherlands approved the experiment.

Phenotyping of the F2 population.

Total antibody titres to SRBC were determined by agglutination in serum from all 672 birds' 1 day before and 5 days after sensitisation (Van der Zijpp and Nieuwland, 1986) at 37 days of age (SRBC primary response) and at 127 days of age (SRBC secondary response). Antibody titres measured against SRBC were expressed as the $\log_2$ of the reciprocal of the highest serum dilution giving complete agglutination. Titres were assessed the same day in 96-well microtiter plates, using SRBC from the same stock as used for the immunisation.

Genotyping of the F2 population.

Genomic DNA was isolated using the Gentra Generation Capture Plate™ Kit from the whole blood according to the protocol provided with the Kit (Gentra Systems, ver. 5.00, Minneapolis, MN, USA). The birds were genotyped for 170 microsatellite markers, equally distributed over the chicken genome, at approximately 20 centiMorgan (cM) intervals. These markers covered GGA1 – GGA19, GGA23, GGA24, GGA27, GGA28, GGAZ and linkage groups E38, E47W24, E60E04W23 (Groenen et al. 2000). PCR reactions were

performed in a total volume of 12 μl containing approximately 10 to 60 ng genomic DNA, 25 mM MgCl2, 50 mM KCl, 10mM Tris-HCl pH = 8.3, 1 mM tetramethylammonium chloride (TMAC), 0.1% Triton X-100, 0.01% gelatin, 200 μM dNTP, 0.25 U Silverstar polymerase (Eurogentech, Seraing, Belgium), 2.3 pmole of each primer and covered with 10 μl mineral oil (Sigma, St. Louis, MO, USA). PCR programme used was: 5 min at 95°C, 35 cycles of 30 seconds at 95°C, 30 seconds at annealing temperatures (45°C - 60°C), and 30 seconds at 72°C, followed by a final elongation step of 4 min at 72°C. PCR products of those markers were pooled in 13 sets in a total volume of 50 μl. A mixture of 1 μl pooled PCR product with 1.6 μl loading buffer (containing 80% formamide and GENESCAN – 350 TAMRA; Applied Biosystems, Perkin-Elmer, Foster City, CA, USA) was loaded on 6% denaturing polyacrylamide gel (Sequagel 6; National Diagnostic, Atlanta, GA, USA), and analysed on ABI 373 Automated Sequencer(Applied Biosystems). Fragment sizes were analysed with GENESCAN (Applied Biosystems) fragment analysis software. Allele calling was performed using GENOTYPER 2.0 software (Applied Biosystems,). All genotypes were checked twice. Finally data was checked for non-inheritance using Cri-Map version 2.4 (Green et al. 1990). All together, 722 animals from generations F0 (32 individuals), F1 (18 individuals), and F2 (672 individuals) were genotyped.

QTL analysis

Statistical analysis.

Prior to the QTL analysis, phenotypic data was adjusted for the systematic hatch effects using the PROC GLM Procedure (SAS Institute, 1995).

$$y_{ij} = \mu + H_i + e_{ij}$$

Where μ is the grand mean, H_i is the effect of the ith hatch (i = 1, 2.... 6) and e_{ij} represents residual effects. The sex effect was not statistically significant, and was therefore omitted from the model.

Regression interval mapping was used for QTL detection. Two different genetic models were used: 1) Paternal half-sib analysis (Knott et al. 1996; De Koning et al. 1999). In this model no assumption was made concerning the allele frequencies in the founder lines and number of QTL alleles. The F2 animals were treated as unrelated half-sib families using the model:

$$y_{ij}= m_i + b_iP_{ij} + e_{ij}$$

Where y_{ij} is the trait score of individual j, originating form sire i; m_i is the average effect for half sib family i; b_i is the substitution effect for a putative QTL; P_{ij} is the conditional probability for individual j of inheriting the first paternal gamete and e_{ij} is the residual effect.
2) Line-cross analysis according to Haley et al. (1994). In this model the power of QTL detection depends on the assumption of fixation of QTL alleles for the trait of interest in the founder lines. This model has been adapted for the detection of parent-of-origin (imprinting) effects containing a paternal, a maternal and a dominance component (De Koning et al. 2000):

$$Y_j = m + a_{pat}p_{patj} + a_{mat}p_{matj} + dp_{dj} + e_j$$

Where m is the population mean, a_{pat} is paternally inherited QTL effect, a_{mat} is maternally inherited QTL effect, d is the dominance effect, p_{patj} is the conditional probability of animal j to inherited allele through its sire, p_{matj} is the conditional probability of animal j to inherited allele through its dam, p_{dj} is conditional probability of animal j to be heterozygous and e_j is the residual error. In the first step, the genome was screened with an imprinting model. For the location with significant evidence of a possible parent-of-origin QTL an additional test was performed. In order to test whether the imprinted model explained the observations better than the mendelian model. This is an F test with 1 d.f. in the numerator and (n-4) d.f. in the denominator (Knott et al. 1998).

Significance thresholds.

Three significance levels were defined: chromosome wide significance which takes into account multiple testing on the specific chromosome; suggestive linkage, i.e. one false positive is expected in a genome scan and genome-wide significance, i.e. 0.05 false positives are expected in a genome scan (Lander and Krugylak, 1995).
Significance thresholds were determined empirically by permutation (Churchill and Doerge, 1994). Data permutation, with at least 10,000 replicates, was used to determine the empirical distribution of the test statistic under the null hypothesis of no QTL associated with the chromosome under study.

Results

Genotyping

In total 170 microsatellite markers were used, out of 208 tested on grandparents of the experimental population. The average coverage of the genome was 78%. The consensus linkage map positions for microsatellite markers (Groenen et al. 2000) were used in the

study; because distances calculated by Two-Point (Cri Map) analysis for the experimental population were very similar to distances for the consensus linkage map.

Half-sib analysis

In the half-sib analysis, two QTL for SRBC primary response were found (Table 1). A suggestive QTL was detected on GGA16 with the F ratio value = 4.47. Two families contribute the most to the QTL with the F ratio value = 12.24 and 10.30 respectively. Second genome wide suggestive QTL was detected on GGA1 with the F ratio value = 3.90. Five families out of 6 contribute equally to this QTL.

Table 1. QTL detected for primary antibody response to SRBC using the half-sib analysis model.

Chromosome	F statistic[1]	QTL variance[2]	Marker bracket
GGA1	3.90**	0.035	*GCT0005 – MCW0049*
GGA16	4.47**	0.040	*MCW0370-MCW0371*

[1] F statistic against H_0 of no QTL

** suggestive linkage

[2] gives the proportion of total variance explained by the QTL

No QTL was detected for SRBC secondary response using the half-sib model.

Line-cross analysis.

In the line-cross analysis, three mendelian QTL were identified (Table 2) for SRBC primary responses. A genome wide significant QTL on GGA7 and two suggestive QTL were detected: one on GGA4 and one on GGA13.

For SRBC secondary responses, suggestive mendelian QTL was detected on GGA18 (Table 3).

Table 3. QTL detected for secondary antibody response to SRBC using the line-cross model.

Chromosome	Effect		Marker bracket	F statistic[1]	QTL variance[2]
	Additive (SE)	Dominance (SE)			
GGA18	0.07 (0.30)	-2.56 (0.71)	*ROS0022 – MCW0219*	6.66**	0.025

[1]F statistic against H_0 of no QTL

** suggestive linkage.

[2]gives the proportion of total variance explained by the QTL .

Under the imprinted model, four non–mendelian QTL were identified for SRBC primary responses (Table 2). First significant QTL was detected on GGA2, with a maternal parent-of-origin effect. Second significant QTL was detected on GGA3, with paternal parent-of-origin effect. Third significant QTL was detected on GGA27, with maternal parent-of-origin effect. A suggestive QTL was detected on linkage group E60E04W23, with a maternal parent-of-origin effect.

No QTL were detected under the imprinted model for SRBC secondary responses.

Table 2. QTL detected for primary antibody response to SRBC using the line-cross analysis model.

Chromosome	Model[1]	*P* value[2]	Additive (SE)	Dominance (SE)	Marker bracket	F statistic[3]	QTL variance[4]
GGA2	Maternal	0.0005	1.31 (0.30)	-	*ADL0114 – ABR0022*	18.58***	0.03
GGA3	Paternal	0.0073	0.98 (0.31)	-	*LE0166 – MCW0037*	9.77***	0.04
GGA4	Mendelian	-	-0.62 (0.43)	3.31 (1.10)	*MCW0005 – ADL0144*	5.79**	0.02
GGA7	Mendelian	-	-0.72 (0.29)	-1.55 (0.57)	*MCW0183 – MCW0236*	7.46***	0.02
GGA13	Mendelian	-	0.91 (0.51)	6.16 (1.83)	*MCW0216 – MCW0315*	7.15**	0.02
E60E04W23	Maternal	0.056	0.75 (0.27)	-		7.86**	0.012
GGA27	Maternal	0.0026	1.14 (0.27)	-	*MCW0300 – MCW0076*	18.07***	0.03

[1]Inferred genetic model used to analyse the data.

[2]*P*-value of the test: Parent-of-origin model against the Mendelian model

[3]F statistic against H_0 of no QTL

** suggestive linkage.

*** genome wide significance at 5% level.

[4]gives the proportion of total variance explained by the QTL

Discussion

We detected QTL influencing the immune response to SRBC in an F2 cross created from lines divergently selected for either high or low antibody response to SRBC. A suggestive QTL detected on GGA16 suggests association with the *MHC* complex, also located on this chromosome. This result is in agreement with the work on Biozzi mice (Puel et al. 1995) which refers to QTL linked to the *MHC* complex. The parental lines that were used to create F2 are different for the *MHC* haplotype. The High line is homozygous for B21, while in the Low line B14 is predominant (Pinard et al.1993). However, in this study the goal was not to establish the effect of *MHC* type on the immune regulation but to screen for QTL spread through out the genome. Also our approach differs from the one used by Puel et al. (1995). They genotyped only the extreme individuals from, the F2, whereas we genotyped the entire F2 population. Selective genotyping only has full power for the selectively genotyped trait (Bovenhuis & Spelman 2000).

Some differences can be observed between the half-sib and the line cross analysis. Higher number of QTL was detected with line cross analysis model. Generally it is believed that the power of a line-cross analysis is more powerful to detect QTL then the half-sib analysis. However, the power of QTL detection under the line cross analysis model depends on allele fixation in the founder lines. If in such a situation the data is analysed using a line cross model, the estimated additive effect will be reduced by a fraction (p_H-p_L), where p_H is the frequency in the H line and p_L is the frequency in the L line. The reduction of this contrast will seriously reduce the power of detecting the QTL using the line cross model (e.g. Alfonso & Haley, 1998 and De Koning et al. 2002). In the extreme case the lines do not differ with respect to the allele frequency and then the power will be equal to 0. However, in that case the QTL still might be detected using a half-sib model. Because both methods revealed different QTL it is not likely that QTL alleles are fixed in the founder populations. This explains why some QTL are not detected in the line cross analysis but show significant effects in the half-sib analysis and the other way around.

Based on the statistical analysis of the selection lines published by Bovenhuis et al. (2002) it can be concluded that in later generations (starting from generation 9) the selection intensity in the low line was not as intense as in high line. In contrast in the high line the selection criterion was maintained at a high level. Therefore, the assumption that at least in the low line the QTL alleles have not been fully fixed due to the reduced selection pressure might be justified. Even when QTL would be fully fixed in the high line, the data for the QTL

analysis may better fit to the half-sib genetic model because of the incomplete fixation in the low line at generation 17th.

Concerning the parent of origin effects, according to the imprinting theory of Moore & Haig (1991) this phenomenon would not occur in birds. Nevertheless, we have statistical evidence for a parent of origin effect at some QTL. The actual biological evidence for parent-of-origin effect in poultry should come from expression studies at the RNA and protein level.

From the results obtained for both primary and secondary SRBC response it could be suggested that there are two sets of genes involved in two stages of SRBC antibody response. Except for QTL located on GGA16 common for primary and secondary response there are regions specific for the primary (GGA1, GGA2, GGA3, GGA4, GGA7 and GGA13) and the secondary (GGA8, GGA18) response to SRBC.

At the moment not many QTL studies for SRBC response in chicken have been performed. In the study of Yonash et al. (2001) a QTL on GGA2 was detected for the primary response to SRBC. There is very significant evidence for a QTL on this chromosome. What confirm this region as related to the primary response to SRBC even within the lines which differ at genetic background. Yonash et al. (2001) used broiler lines, while this study used laying hens.

Because of the complex nature of the SRBC antigen, and the results of the experiment on Biozzi mice (Puel et al. 1996) we expected to identify a higher number of QTL. In the F2 analysis of the Biozzi mice (Puel et al. 1995) about ten genes were implicated to play a role of some significance in the regulation of the Ab response to SRBC. Among these genes were the *MHC, Immunoglobulin heavy and light genes (Igh, Igl)* and *T cell receptor (TCR)* gene, which are obvious candidate genes with well-known functions in the adaptive immune response. In the experimental population that we used, QTL was detected on GGA16 (*MHC*) indicate a role of the MHC in the responses to both primary and secondary SRBC challenge. Also around the location of *T cell receptor beta chain* (GGA1) and *Interferon gamma* (GGA1) QTL for SRBC primary response was identified.

Although there are candidate genes available, the confidence interval is still large. A next step in QTL mapping experiments is narrowing down the QTL interval, which can be done by using additional markers. New markers for instance single nucleotide polymorphism (SNP) markers will have to be developed in order to create a higher density of informative markers.

Besides adding new markers in the F2 population creating additional generations (F3, F4) will improve fine mapping of QTL for antibody response to SRBC. In addition the sequence of the chicken genome in 2004 will future improve the search for candidate genes.

Our study aimed at the identification of QTL involved in the regulation of the immune response in chicken selected for SRBC primary response. Based on the different identified QTL a difference in the genetic control of primary and secondary immune response to SRBC is implicated. In conclusion, our results suggest, that beside the *MHC* several other genes are involved in regulation of immune responses to SRBC.

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CHAPTER 3

Detection of different QTL for antibody responses to Keyhole Lympet Hemocyanin and *Mycobacterium butyricum* in two unrelated populations of laying hens.

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ABSTRACT Quantitative trait loci involved in the primary antibody response to keyhole lympet hemocyanin (KLH) and *Mycobacterium butyricum* were detected, in two independent populations of laying hens. The first population was an F_2 cross (H/L) of lines divergently selected for either high or low primary antibody responses to SRBC, and the second population is an F_2 cross between two commercial layer lines displaying differences in feather pecking behavior (FP). Both populations were typed with microsatellite markers widely distributed over the genome, with similar intervals between markers. Titers of antibodies binding keyhole lympet hemocyanin (KLH) and *M. butyricum* were measured for all individuals by ELISA. Two genetic models were applied to detect QTL involved in the humoral immune response: a half-sib model, and a line-cross model both using the regression interval method. In the half-sib analysis, two QTL (on GGA14 and GGA3) were detected for the antibody response to KLH for the H/L population and three QTL (GGA12, GGA14 and GGA16) were detected for the FP population. Two QTL were detected for *M. butyricum* on GGA14 and GGA18 in the FP population using the half-sib analysis model. Three QTL were detected for the H/L population on GGA3, GGA13 and GGA14 using the line-cross analysis model. A QTL for the primary antibody response to KLH detected on GGA14 was validated in both populations under the half-sib analysis model. The present data suggest differences in the genetic regulation of antibody responses to two different T-cell dependent antigens.

Key words: selection, antibody, chicken, microsatellite, quantitative trait loci

Introduction

Improvement of health of chickens by selection for enhanced general resistance to pathogens is an attractive alternative for veterinary health treatments. However, there are many pathogenic organisms to which poultry may be exposed during a lifetime. Also, it is difficult to identify infectious organisms that could serve as the appropriate selection targets (Lamont, 1998). Genetic resistance of poultry may be improved by selecting for a generalized improved immune response by selection for immune responses to a complex antigen such as SRBC. Chickens divergently selected for high antibody responses to SRBC showed higher resistance to some infectious diseases, e.g., Marek's Disease (Pinard et al., 1992) and coccidiosis (Parmentier et al., 2001). Similar results were found with various other selection lines: however, enhanced resistance to one infection could be accompanied with enhanced susceptibility to other infections (Gross et al., 1980; Dunnington et al., 1986). Divergent selection to antigens (e.g. SRBC, *E. coli*) resulted in divergent humoral and cellular immune responses to several other antigens, e.g., *Brucella abortus* (Nelson et al., 1995), *Escherichia coli* (Pitcovski et al., 2001; Yunis et al., 2002), bovine serum albumin (Parmentier et al., 1994), *Mycobacterium butyricum* (Parmentier et al., 1998), concanavalin A (Parmentier et al., 1993, Kreukniet et al., 1994), phytohemagglutinin (Pinard 2002), and vaccines (Parmentier et al., 1996) in various selection lines. These results suggested that divergent selection for one antigen e.g. SRBC may influence regulation of the humoral as well as cellular immune responses for different types of antigens.

A well established chicken genetic map enables the use of molecular tools to detect loci affecting complex traits, in which one to one relationships between phenotype and genotype do not exist. QTL experiments in poultry dealing with immune traits focussed on Marek's disease virus (Vallejo et al., 1998), antibody responses to *Salmonella enteritidis* vaccine (Kaiser et al. 2002), and antibody responses to *E. coli* vaccine (Yonash et al., 2001; Yunis et al., 2002).

Different QTL for primary and secondary antibody responses to SRBC in the reciprocal F_2 cross of the H/L selection lines have been described (M. Siwek, unpublished data). In the present study, keyhole lympet hemocyanin (KLH) and *M. butyricum* were used as antigens to detect QTL underlying primary antibody responses to other T-cell dependent antigens. The KLH antigen is an antigen that birds do not encounter during their lifetime, and thus represents a novel antigen, which is suitable to measure primary immune responses. In mammals, KLH immunisation resulted in TH-2 dependent (antibody) immune responses

(Bliss et al., 1996). On the other hand, *M. butyricum* is a specific antigen that induces TH-1 immune responses in mammals (Mossann and Sad, 1996). The purpose of the current study was to detect QTL controlling antibody responses to antigens other then than the one used for selection (SRBC) in the H/L lines and to QTL affecting antibody response to both antigens. In addition, it is important to confirm results from an initial QTL genome scan of a population in an independent study to be sure that QTL detected were not artefacts. Such confirmation studies should be independent of the original QTL experiment and different animals should be used rather then those in the original QTL study (Spelman and Bovenhuis, 1998). Here the results of such a QTL analysis aiming at the identification of QTL involved in regulation of antibody response to KLH and *M. butyricum* are presented in two independent populations of laying hens.

Material and methods.

Chicken Populations.

The first population was the H/L F_2 population that originated from a cross (ISA Warren, medium heavy layers) between two divergently selected lines for either high (H line) or low (L line) primary antibody response to SRBC. Selection was based on the individual antibody titer at 5 d after primary intramuscular immunisation with SRBC at 37 d of age (Van der Zijpp and Nieuwland, 1986). Reciprocal crosses with birds from the 18th generation were made to generate F_1 animals. From the F_1 generation, an intercross was made to produce 672 individuals in 6 hatches of the F_2 experimental population.

The second population was the feather pecking (FP) F_2 population which was created from a cross between two commercial lines of layers as described by Buitenhuis et al. (2003). In brief, reciprocal crosses were made to create F_1 animals. Seven half-sib families were created to obtain 630 F_2 animals. The F_2 chicks were hatch in 5 hatches at 2 wk intervals.

For both populations all birds were housed in brooder cages with free access to water and feed (152 g/kg CP and 2,817 kcal/kg ME). Birds were not beak-trimmed and each individual bird was marked with a wing-band. All birds were vaccinated against Marek's disease, infectious bronchitis, and infectious bursal disease at hatch, and 2 and 15 d of age, respectively. The Ethical Committee On Animal Care and Welfare of Wageningen University, The Netherlands approved the experiment.

Phenotyping of the F_2 H/L and FP Populations.

Total antibody responses to KLH were measured in individual plasma samples obtained at 7 d after s.c. immunization with 1 mg KLH[1] in 1 mL PBS (pH 7.2) at 12 wk of age for the H/L

population, and at 36 wk of age for the FP population. Antibody responses to *M. butyricum*[2] were measured in individual plasma samples at 11 d after s.c. immunisation with 1 mg *M. butyricum* in 1 mL PBS at 14 wk of age for the H/L population, and at 39 wk of age for the FP population. Antibody titers to KLH and *M. butyricum* of all birds were measured by an indirect ELISA as described by Sijben et al. (2000). Titers were expressed as the $\log_2$ values of the highest dilution giving a positive reaction.

Genotyping of the F_2 H/L and FP Populations.

Genomic DNA was isolated using the Gentra Generation Capture Plate™ Kit[3] from the whole blood according to the Capture Plate™ Kit protocol[3]. In total, 170 and 180 microsatellite markers were chosen, for the H/L and FP populations respectively. These microsatellite markers were widely distributed over the chicken genome, approximately 20 cM apart. These markers were covering GGA1 – GGA19, GGA23, GGA24, GGA27, GGA28, GGAZ, and linkage groups E38, E47W24 and E60E04W23 (Groenen et al., 2000). The PCR reactions were performed as described by Crooijmans et al. (1997). The PCR program used was: 5 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at annealing temperature (45°C to 60°C), and 30 s at 72°C followed by a final elongation step of 4 min at 72°C. Markers were divided over 13 sets.

For each set PCR products of these markers were pooled per individual in a total volume of 50 µL. Finally 1 µL of pooled PCR product was mixed with 1.6 µL loading buffer (containing 80% formamide and GENESCAN – 350 TAMRA marker[4]) loaded on 6% denaturing polyacrylamide gel (Sequagel 6[5]) and analysed on ABI 373[4]. Fragment sizes were analysed with GENESCAN fragment analysis software[4] and allele identification was performed using GENOTYPER 2 software[4].

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[5] National Diagnostic, Atlanta, GA

All genotypes were checked twice. Finally the data was checked for non-Mendelian inheritance using the CRI-Map (Green et al., 1990). All together, 722 birds from generations F_0, F_1, and F_2 of the H/L population, and 689 from generations F_0, F_1 and F_2 of the FP population were genotyped.

QTL Analysis

The analyses were performed separately for both experimental populations.

Prior to the QTL analysis, phenotypic data were adjusted for the systematic hatch effects using the PROC GLM Procedure (SAS Institute, 1995).

$$Y_{ij} = \mu + H_i + e_{ij}$$

Where μ is the grand mean, H_i is the effect of the i_{th} hatch (i = 1, 2,.... , 6 for the H/L population and i = 1, 2,.... , 5 for FP population respectively) and e_{ij} represents residual effects. The hatch is referred to a group of individuals hatched at one time.

The sex effect was not statistically significant and was, therefore, omitted in the model.

Regression interval mapping was used for QTL detection. Two different genetic models were used: 1) paternal half-sib analysis (Knott et al., 1996; De Koning et al., 1999) and 2) line-cross analysis model (Haley et al., 1994). In the paternal half-sib model no assumptions are made concerning the allele frequencies in the founder lines and number of QTL alleles. The F_2 animals are treated as number of unrelated half-sib families using the model:

$$Y_{ij} = m_i + b_i P_{ij} + e_{ij}$$

where Y_{ij} is the trait score of individual j, originating from sire i; m_i is the average effect for half-sib family i; b_i is the substitution effect for a putative QTL; P_{ij} is the conditional probability for individual j of inheriting the first paternal gamete, and e_{ij} is the residual effect.

In the line-cross model, the power of QTL detection depends on the assumption of fixation of QTL alleles for the trait of interest in the founder lines. In this model the alternative alleles at the QTL are traced back to the founder lines. At every centi Morgan across the genome the following model is fitted:

$$Y_j = m + ax_{aj} + dx_{dj} + e_j$$

where Y_j is the adjusted trait score of animal j, m is the population mean, a and d are the estimated additive and dominant effect of a putative QTL at the given location, x_{aj} is the conditional probability of animal j carrying both alleles from the same line, x_{dj} is the conditional probability of being heterozygous at given location, and e_j is the residual error.

Significance Thresholds

Significance thresholds were determined empirically by a permutation test (Churchill and Doerge, 1994). Data permutation, with at least 10,000 replicates, was used to determine the empirical distribution of the test statistic under the null hypothesis of no QTL associated with the chromosome under study (De Koning et al., 1999). Three significance levels were defined according to Lander and Krugylak (1995) were 1) chromosome wide linkage (which take into account multiple testing on the specific chromosome) 2) suggestive linkage (one false positive is expected in a genome scan); and 3) significant linkage (statistical evidence expected to occur 0.05 times in a genome scan).

Results

Phenotypic Measurements

In both populations antibody titers to KLH and *M. butyricum* were not normally distributed. The mean titer $\pm$ SD at 7 d after immunization for KLH in the H/L population was 5.10 $\pm$ 2.12, while in the FP population the mean titer of antibodies binding KLH was 6.42 $\pm$ 1.40. Mean titers of antibodies binding ± SD at 11d after immunization for *M. butyricum* were 4.23 $\pm$ 2.09 and 7.55 $\pm$ 1.46 for the H/L and FP populations, respectively.

Genotyping

In total, 208 microsatellite markers were tested on the H/L and FP grandparents for polymorphism. Thirty-eight markers for H/L and twenty-eight markers for FP could not be used in the total genome scan either because markers did not amplify, or the markers were not informative in the cross. The map distances in general did not differ much from the distances on the consensus linkage map therefore the map distances based on the consensus map were used. The estimated genome coverage is 78% for H/L population and 80% for FP population.

Half-Sib Analysis

H/L Population. The results found for the KLH antibody response using the half-sib analysis are presented in Table 1.

1. **Table 1.** QTL for antibody response to keyhole lympet hemocyanin (KLH) and Mycobacterium butyricum using the half-sib analysis model in the H/L[1] population.

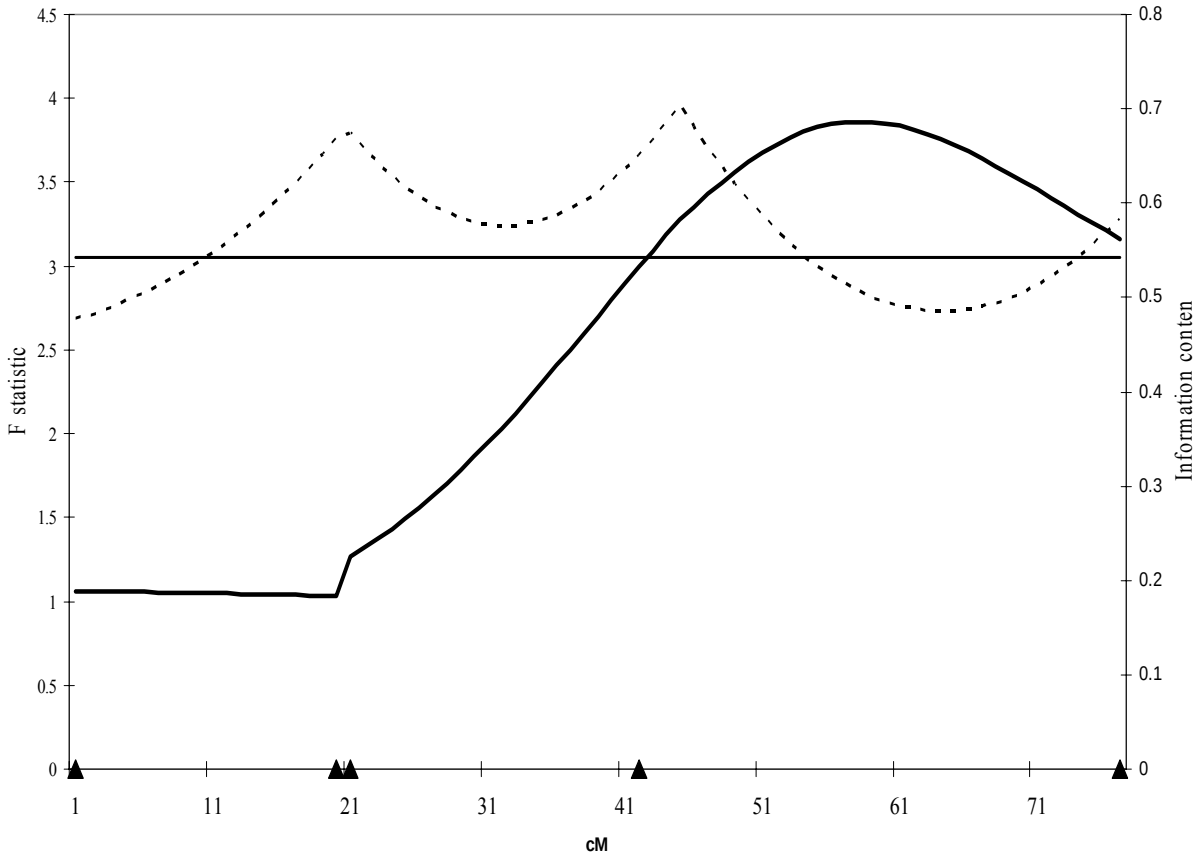
Trait	Chromosome	Marker bracket	F ratio[2]
KLH	GGA3	MCW0261 – MCW0169	3.22†
	GGA14	MCW0123 – MCW0225	3.86†
M. butyricum	GGA4	MCW0114 – MCW0005	4.98*

[1]H/L = F_2 cross of high and low lines

[2] †suggestive linkage; *genome wide linkage at 5% level

For the antibody response to KLH two suggestive QTL were detected. First QTL with an F ratio of 3.86 was detected on GGA14 between marker brackets MCW0123 and MCW0225 (Figure 1). Family 3 contributed the most to this QTL with an F ratio of 18.78 and an allele substitution effect of 2.19.

Figure 1. Test statistic for GGA14 with regard to the antibody response to keyhole lympet hemocyanin (KLH) under the half-sib analysis model for the F_2 cross of high and low line (H/L) population. The solid curve describes the test statistic for KLH and the dotted line describes information content. The solid horizontal line indicates 5% genome wide threshold. Triangles on the x axis indicate position of microsatellite markers.



The contribution of individual family QTL effects with regards to GGA14 is presented in Table 2.

Table 2. Overview of estimated QTL effects within families for keyhole lympet hemocyanin response with regard to GGA14

	H/L population[1]	
Family	QTL effect	SE
1	0.17	0.50
2	0.09	0.42
3	2.19	0.50
4	-0.16	0.38
5	0.43	0.30
6	0.30	0.47
	FP population[2]	
Family	QTL effect	SE
1	0.41	0.37
2	0.42	0.31
3	1.29	0.66
4	0.00	0.22
5	0.31	0.27
6	-0.37	0.23
7	1.82	0.50

[1]H/L = F_2 cross of high and low lines.

[2]FP = F_2 cross of feather pecking.

Second QTL with an F ratio of 3.22 was detected on GGA3 between marker brackets MCW0261 and MCW0169 (Table 1). Family 5 contributed the most to this QTL with an F ratio of 10.35 and an allele substitution effect of 1.31. For antibody response to *M. butyricum* a suggestive QTL with an F ratio of 4.98 was detected on GGA4 in a marker bracket MCW0114 and MCW005. Family 4 contributed the most to this QTL with an F ratio of 22.57 and an allele substitution effect of -1.34.

FP Population. The QTL detected for the KLH antibody response using the half-sib analysis are presented in Table 3.

Table 3. QTL for antibody response to keyhole lympet hemocyanin (KLH) and *Mycobacterium butyricum* using the half-sib analysis model in the FP[1] population**.**

Trait	Chromosome	Marker Bracket	F ratio[2]
KLH	GGA12	ADL0372 – MCW0198	2.47#
	GGA14	MCW0296 – ADL0118	4.08*
	GGA16	MCW0370 – MCW0371	1.95#
M.butyricum	GGA14	MCW0296 – MCW0136	2.50#
	GGA18	ROS0022 – MCW0219	1.28#

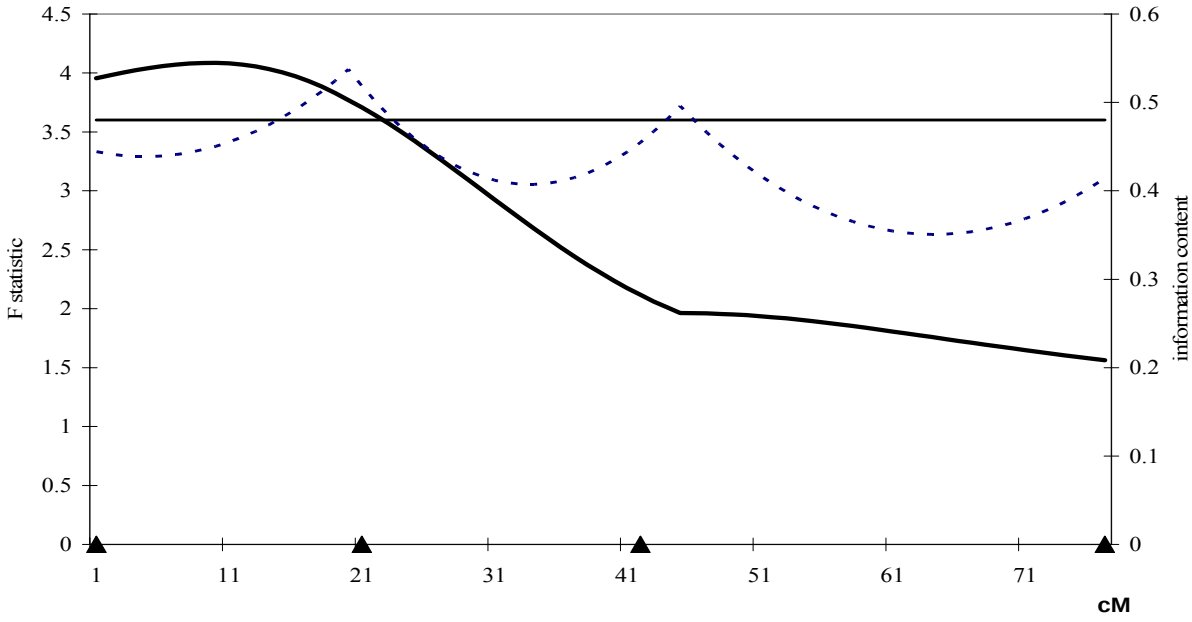
[1]FP = feather pecking.

[2]# chromosome wide linkage; *genome wide linkage at 5% level**.**

For the antibody response to KLH three QTL were detected. A significant QTL with an F ratio of 4.08 was detected on GGA14 between marker brackets MCW0296 and MCW0123 (Figure 2). Two families contributed the most to this QTL. Family 7 with an F ratio of 12.97 and an allele substitution effect of 1.82 and family 3 with an F ratio of 3.72 and an allele substitution effect of 1.29. The family contribution to QTL on GGA14 is presented in Table 2. A chromosome wide QTL with an F ratio of 2.47 was detected on GGA12 between marker brackets ADL0372 and MCW0198 (Table 3). Second chromosome wide QTL with an F ratio of 1.95 was detected on GGA16 between marker bracket MCW0370 and MCW0371.

For the antibody response to *M. butyricum* two chromosome wide QTL were detected. First QTL was detected on GGA14 with an F ratio of 2.50 between marker brackets MCW0296 and MCW0136 (Table 3). Second QTL was detected on GGA18 with an F ratio of 1.28 between marker brackets ROS0022 and MCW0219.

Figure 2. Test statistic for GGA14 with regard to the antibody response to keyhole lympet hemocyanin (KLH) under the half-sib analysis model for the F2 cross of feather pecking line (FP) population. The solid curve describes the test statistic for KLH and the dotted line describes information content. The solid horizontal line indicates 5% genome wide threshold. Triangles on the x axis indicate position of microsatellite markers.



Line-Cross Analysis

There were no QTL detected for antibody response to both KLH and *M. butyricum* using the line-cross analysis model in the FP population. For the H/L population the results found for the KLH antibody response presented in Table 4.

Table 4. QTL affecting immune response to keyhole lympet hemocyanin (KLH) in the H/L population detected using the line-cross analysis model.

Trait	Chromosome	a[1] (SE)	d[1] (SE)	Marker Bracket	F ratio
KLH	GGA3	1.56 (0.38)	-3.06 (2.20)	MCW0162 – MCW004	9.82*
	GGA13	0.76 (0.26)	-1.44 (0.85)	MCW0340 – MCW0214	0.37†
	GGA14	1.33 (0.35)	0.20 (1.13)	MCW0123 – MCW0225	0.41†

[1]Estimated QTL effects for the genetic model: a is the additive effect; and d is the dominance level.
† suggestive linkage; * genome wide linkage at 5% level.

For the antibody response to KLH three mendelian QTL were detected. A significant QTL was detected on GGA14 between marker bracket MCW0123 – MCW0225, with dominance effect of 0.20 and additive effect of 1.33. A suggestive QTL was detected on GGA3 between marker brackets MCW0162 and MCW0004, with a dominance effect of –3.06 and an additive effect of 1.56. Second suggestive QTL was detected on GGA13 between marker brackets MCW0340 and MCW0214, with a dominance effect of –1.44 and an additive effect of 0.76. There were no QTL detected for antibody response to *M. butyricum* using the line-cross analysis model (data not shown).

An overview of QTL related to the primary responses to different antigens, and comparing QTL detected in both experimental populations, using half-sib analysis model is presented in Table 5.

Table 5. QTL affecting primary immune response to SRBC, keyhole lympet hemocyanin (KLH), and *Mycobacterium butyricum* in the H/L[1] and FP[2] populations detected using the half – sib analysis model.

Population	Antigen	Chromosome					
H/L	SRBC	1				16^{B}	
	KLH		3		14^{A}		
FP	KLH			12	14^{A}	16^{B}	
	M. butyricum				14^{A}		18

[1]H/L = F_2 cross of high and low lines.
[2]FP = F_2 cross of feather pecking.
[A-B] Common superscript letters indicate common chromosomes for different antigens in both populations.

First, there were chromosomes dedicated to specific antigens: QTL for primary antibody responses to SRBC was detected on GGA1 in the marker bracket GCT0005 – MCW0049 (M. Siwek, 2003 Wageningen University, Wageningen, The Netherlands, personal communication). A QTL restricted for primary antibody responses to KLH was detected on GGA3. Second, some common chromosomes were detected for combinations of antigens. On GGA14 a QTL was located for antibody response to KLH and *M. butyricum.* On GGA16 a QTL was located for antibody response to SRBC and KLH.

Discussion

In the current study results are presented of a QTL mapping analysis for primary antibody responses to KLH and *M. butyricum* in two independent F_2 populations. The first F_2 population was a cross of chicken lines divergently selected for either high or low antibody responses to SRBC. The second population was a cross between two commercial layer lines. Both experimental populations were typed with microsatellite markers widely distributed over the genome, with similar intervals between markers.

To date, there are not many QTL studies performed dealing with immune responses of poultry. Those, which have been performed, are generally characterised by low numbers of F_2 individuals (Yonash et al. 1999), or the F_2 individuals were analyzed together with individuals from a back cross (Yonash et al. 2001). Thresholds levels applied in these experiments were mainly chromosome wide, following the guidelines of Lander and Kruglyak (1995), and did not take into account testing the whole genome. Because of the different analysis methods applied it is difficult to compare the results from other experiments with those of the current study. To verify the existence of a QTL observed in an initial genome scan, further confirmation is necessary, preferably on independent populations as described by Spelman and Bovenhuis (1998). Here the detection of a QTL for primary antibody responses to KLH is presented, which has been mapped in two independent chicken populations. In both populations, H/L and FP, all methods used for analysis were the same: simple regression interval mapping, multiple testing, and thresholds following the guidelines of Langer and Kruglyak (1995), which allowed comparison of results from the two experiments. All individuals from both populations received the same treatment. The influence of the vaccination on antibody titers cannot be excluded but such an effect was not observed in the parental lines of H/L populations. Also both experimental populations followed the same routine vaccination schedule, where the last vaccine was injected at day 28. The cross influence of the antigens may be excluded. For every antigen and every test day, the zero titers were measured. The variation observed for day zero titers was very small. The cross epitopes for specific antigens are not known.

A QTL for the antibody response to KLH detected on GGA14 for the H/L population was validated in an independent FP population. The QTL detected in the FP population showed a higher threshold than the one found for the H/L population. However, the effects of that QTL were not very strong in both cases. There was only one family contributing to this QTL in the H/L population, and two families in the FP population. The observed independent

confirmation of this QTL motivates further investigations in this region and eventually positional cloning.

Current QTL validation experiments have focussed on rodents (McClearn et al., 1998; Radcliffe et al., 2000; Erwin et al., 2001; Klein et al., 2001; Bergeson et al., 2001) and dairy cattle (Arranz et al., 1998). To the authors knowledge, the present study is the first confirmation with regards to a QTL of an immune related trait in chickens.

Although the QTL detected using the half-sib analysis and the line-cross analysis model are located in the same marker bracket it is not very likely that QTL alleles are fixed for this trait. There are a limited number of families which contributed to these QTL in the H/L population therefore; most likely the QTL alleles with relatively large effect but low frequency were detected. In the present study primary antibody responses to either KLH or *M. butyricum* were measured for the three following reasons. Firstly, in mammals *M. butyricum* (TH-1) and KLH (TH-2) reflect different types of antibody responses. Secondly, significant line differences were found with respect to antibody titres to KLH (data not published), and *M. butyricum* (Parmentier et al., 1998) in the founder lines of the current H/L population antibody responses to both antigens being always higher in the H line. Thirdly, although the TH-1/TH-2 paradigm has formally not been established yet in poultry, differences between the antibody responses to these two antigens, with respect to magnitude, kinetics, and modulation, were described by Sijben et al. (2002) suggesting different processing of the two antigens. In this respect it was noteworthy that both types of T-cell dependent antibody responses to KLH and *M. butyricum* were not reflected by corresponding QTL in both chicken populations.

From the overall analysis of QTL detected for primary antibody response to SRBC, KLH, and *M.butyricum* a conclusion about 'private' and 'public' chromosomal regions related to mentioned antigens was made. First 'private' chromosomes dedicated to specific antigens: QTL for primary antibody responses to SRBC was detected on GGA1. A QTL restricted for primary antibody responses to KLH was detected on GGA3. Second, some 'public' chromosomes were detected for combinations of antigens. On GGA16 a QTL was located for antibody response to SRBC and KLH. On GGA14 a QTL was detected for antibody response to KLH and *M. butyricum.* These shared QTL suggested corresponding (public) genes underlying non-antigen specific pathways of antibody responses to different T-cell dependent antigens. Similarly, the QTL restricted to single antigens, when confirmed in an independent fashion as shown in the current study for GGA14, might represent 'private' antigen specific pathways of the antibody response. The differences of QTL detected

between the populations, or the absence of QTL in the FP population versus the presence in the H/L population may also be related to the differences in age at immunization between the lines, the FP line being older and showing higher titers, or alternatively, the immunological features of the antigenic. For instance, QTL detected in the H/L population for primary antibody responses to SRBC did not coincide with those for the KLH primary antibody response. This might be explained by the different ages at immunization, however, the agglutinating primary antibody response to SRBC consists mainly of the IgM isotype, whereas primary responses to KLH in the SRBC-selection lines are also composed of IgG antibodies. Also little knowledge is available of the optimal moment of antibody responses in the various F_2 populations. In the present study, antibody titers were measured at a single moment after sensitisation.

In the chicken, several genes involved in regulation of immune responses were placed on the genetic map. Some of the major genes were mapped on GGA1 (γ-interferon; Guttenbach et al., 2000), GGA15 (immunoglobulin light chain; Zhao et al., 2000), GGA16 (B-region; Dominguez-Steglich et al., 1991) and GGA27 (T cell receptor α chain; Wang et al., 1997). Interestingly, QTL was found on GGA16 for the primary antibody responses to KLH in the H/L population. The current founder H line almost exclusively consists of the BG-21 haplotype, whereas the founder L line is almost exclusively of the BG-14 haplotype. As yet GGA16 requires further coverage by polymorphic DNA markers, however, primary antibody responses to SRBC were reflected by a QTL on GGA16 (M. Siwek, unpublished data). This suggests that the B locus might be major region underlying the variability of primary antibody responses to T-cell dependent antigens. At present, there are no known genes mapped in the region of the validated QTL on GGA14 that are related to immune responses. To obtain a high-density comparative map many more genes have to be located. That can be achieved using the Wageningen Bacterial Artificial Chromosome (BAC) library and identifying new genes on the chicken genome (Crooijmans et al., 2000; 2001; Jennen et al., 2002; Buitenhuis et al., 2002). Apart of the BAC library, it is also possible to use, information provided by chicken radiation hybrid panel as a powerful method in a comparative mapping (Morisson et al., 2002). The availability of sequenced chicken cDNAs (http://www.chick.umist.ac.uk Boardman et al., 2002, Abdrakhmanov et al., 2002) is indispensable to improve the chicken gene maps in general and especially in those chromosomal regions where QTL have been identified. Next to the improvement of the comparative map it is important to verify the QTL in subsequent generation to reduce the QTL region.

In the same F_2 H/L birds as described in the current paper, different QTL for primary and secondary antibody responses to SRBC were detected. Regions with major immune response genes, such as the B-region (GGA16), and TCRA (GGA27) were only indicated for the primary antibody response to SRBC (M. Siwek, unpublished data). Currently, an F_4 generation of the H/L SRBC selection population has been bred.

In summary, the result of a QTL analysis aiming at the identification of QTL involved in the primary antibody response to KLH and *M. butyricum* was presented for two independent populations of laying hens. The study suggested that different molecular mechanisms are involved in the primary humoral response to different T-cell dependent antigens, e.g. SRBC, KLH, and *M. butyricum* in chickens. The presence of a QTL on GGA14 for the primary antibody response to KLH was validated in two independent populations.

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CHAPTER 4

Detection of QTL for innate: non specific antibody levels binding LPS and LTA in two independent populations of laying hens.

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Abstract In the current study results are presented of an experiment dealing with the Natural antibodies which are measured by level of homotopes LPS and LTA. In total 12 QTL were detected to non-specific antibody titers directed to LTA and LPS and at 2 ages after applying 2 statistical models in an F2 population descendant from a cross between chickens divergently selected for either High or Low specific Ab responses to SRBC. For antibody level binding LPS: QTL on GGA1, GGA4 (both ages), GGA3, GGA8, GGA12 and GGA18 were detected. For antibody level binding LTA QTL on GGA2 (both ages), GGA3 (both models), and QTL on GGA10 were detected. Similarly in an FP cross overall seven QTL were detected. For antibody level directed to LTA QTL on GGA3, GGA7, two QTL for GGA14 (both models) were detected. For LPS QTL on GGA8, GGA18 and GGA27 were detected. In conclusion: different QTL regions are associated with immune responses to homotopes LPS and LTA in poultry.

Keywords Natural antibodies, quantitative trait loci, laying hens

Introduction

Immune defence consist of “innate” and “adaptive” immunity. The innate immune system predates the adaptive immune system on at least two levels. First, various components of the innate immune system are present in all multicellular organisms, whereas adaptive responses are found only in vertebrates. Second, the innate immune system uses receptors either cell bound or soluble which are ancient in their origin [1]. Innate immunity is represented by various natural barriers such as skin, physiological factors such as pH, temperature, oxygen tension, lysozyme and complement and phagocytic cells. Often the innate immune system can terminate infections before the adaptive immune system is activated. The “specific” parts of the innate immune system are formed by natural antibodies (NAb) which are present in non-immunised individuals. NAbs do not require induction of B-cells by on purpose antigenic challenge or mitogenic stimulation. NAb have low binding affinity and a broad specificity repertoire [2, 3]. In mammals, NAb are mainly of the IgM isotype, however, also IgA and IgG have been reported [4, 5] and are probably involved in early clearance of foreign material. The amount of NAbs increases with the age of the individual. The level of NAbs may be enhanced by either polyclonal stimulation by exogenous microbes by initiating responses of auto-reactive B cells or correspond with the secretion of naturally occuring auto – reactive B cell clones. NAbs probably enhance processes of antigen uptake and antigen presentation via dendrites or B cells. Specific immunity and protection may be also enhanced by NAbs and idiotype – antiidiotype networks [5, 6]. It is very likely that NAbs are present in chickens [7]. To date, chicken antibodies binding ovarian antigens [8] and MHC class IV were reported [9, 10]. NAbs were detected in chicken lines selected for either high or low primary (specific) antibody (SpAb) responses to SRBC [11]. Higher NAb levels were found in the line genetically selected for high (specific) antibody response to SRBC as compared to the line genetically selected for low (specific) antibody responses. Furthermore adaptive transfer of Nabs enhanced specific Ab response in recipient [12] indicating either a functional or genetic relationship between Nab and SpAb or both.

Lipoteichoic acid (LTA) and lipopolysaccharide (LPS) act as homotopes. Homotopes are microbial stimulators of the innate immune system. LTA is shared by gram positive bacteria, whereas LPS is shared by gram negative bacteria. Specific cellular and humoral immune responses depend on the previous activation of the innate immune system. The crucial role of homotopes is polarization of the specific immune system. Homotopes bind to Toll – like receptors (TLR’s), receptors of Heat-shock proteins (HsP), complement

component C3-receptors, and Fcγ - receptors on phagocytes and Natural Killer cells which act as pattern recognition receptors (PRR). In general, PRR are expressed on cells of the innate immune system that first encounter patogens during infection. In mammals, homotopes upon binding to PRR induce expression of different cytokines such as tumor necrosis factor (TNF) - α, IL-6, IL-12, IL-15 and type 1 interferons by Antigen Presenting Cells (APC). LPS induce IL-10 release, and enhance expression of ICAM-1, CD119 (γ-interferon receptor) and MHC molecules on epithelial cells [14], whereas LTA induce the release of other cytokines like TNF-α, IL-2, IL-1β, IL-6, IL-12, IL-18, and enhance antibody responses to parentally administered antigen [13, 14, 15, 16]. In general, LPS acts through binding to TLR4 and inducing the release of IL1 or INFγ and is associated with TH1 type of inflamatory responses. Whereas LTA acts through binding to TLR2 and induces the release of IL4, IL13 stimulating TH2 type of antibody responses.

The availability of a genetic map in chicken [17] enables the use of molecular genetics as analytical tools to detect loci affecting complex traits, in which a one to one relationship between phenotype and genotype does not exist. Loci affecting these complex traits are quantitative trait loci (QTL).

QTL for primary and secondary antibody responses to SRBC in the reciprocal F_2 cross of the H and L selection lines have been already described [18]. Additional QTL for primary (specific) antibody responses to KLH (keyhole lymphet hemocyanin) and *M.butyricum* in two unrelated populations have been detected [19]. QTL for specific primary Ab responses to SRBC, KLH and *M.butyricum* do not coincide [18, 19], indicating that the specific Ab response to these antigens pathway specific genes play a role in the regulation of these responses. The innate immunity is thought to be the initiator of the specific immune response, the current research was focussed on the innate/adaptive immunity relation by addressing following questions:

1. Can QTL for Nabs level be detected????
2. When QTL are detected, do they differ for different homotopes and at different age?
3. What is the relation between QTL for NAbs to SpAb?

To answer these question an experiment was set up in which three points were taken into consideration: 1. Ab responses were measured to two different homotopes (LTA and LPS), which are known to stimulate different pathway of adaptive immune responses and to which high levels of antibody levels are present in chickens [11] 2. two unrelated chicken populations with different genetic background: one selected for specific antibody responses (

H/L population) the second selected for production traits (FP population) were used 3. Ab responses were measured at different ages to establish the age influence on the level of NAbs.

To the authors' knowledge, the presented study is the first one, dealing with QTL influencing NAbs levels in chickens.

Material and Methods

Chicken Populations

The first population was the H/L F_2 population that originated from a cross (ISA Warren, medium heavy layers) between two divergently selected lines for either high (H line) or low (L line) primary antibody response to SRBC. Selection was based on the individual antibody titer at 5 d after primary intramuscular immunisation with SRBC at 37 d of age [20]. Reciprocal crosses with birds from the 18th generation were made to generate F_1 animals. From the F_1 generation, an intercross was made to produce 672 individuals in 6 hatches of the F_2 experimental population.

The second population was the feather pecking (FP) F_2 population which was created from a cross between two commercial lines of layers as described by Buitenhuis et al. [21]. In brief, reciprocal crosses were made to create F_1 animals. Seven half-sib families were created to obtain 630 F_2 female animals. The F_2 chicks were hatched in 5 hatches at 2 wk intervals.

For both populations all birds were housed in brooder cages with free access to water and feed (152 g/kg CP and 2,817 kcal/kg ME). Birds were not beak-trimmed and each individual bird was marked with a wing-band. All birds were vaccinated against Marek's disease, infectious bronchitis, and infectious bursal disease at hatch, and 2 and 15 d of age, respectively.

Phenotyping of the F_2 H/L and FP Populations.

The Ab level of LPS and LTA in serum from all the birds was determined by ELISA at 5 wks and 18 wks of age for H/L population and 38 wks of age for FP population. Briefly, 96 well plates were coated with 4 µg/mL of LPS or 10 µg/mL LTA. After subsequent washing with tap water, 0.05% Tween, the plates were incubated with serial dilution of serum. Binding of Ab to LPS and LTA antigen was detected using 1:20,000 diluted rabbit anti-chicken IgG_{H+L} coupled to peroxidase. After washing, tetramethylbenzidine and 0.05% H_2O_2 were added and incubated for 10 minutes at room temperature. The reaction was stopped with 2.5N H_2SO_4 . Extinctions were measured with Multiscan at wavelength of 450

nm. Titers were expressed as the $\log_2$ values of the highest dilution giving a positive reaction.

Total antibody responses to KLH were measured in individual plasma samples obtained at 7 d after s.c. immunization with 1 mg KLH (Difco Laboratories, Detroit, USA) in 1 mL PBS (pH 7.2) at 12 wk of age for the H/L population, and at 36 wk of age for the FP population. Antibody titers to KLH of all birds were measured by an indirect ELISA as described by Sijben et al. [22]. Titers were expressed as the $\log_2$ values of the highest dilution giving a positive reaction.

Genotyping of the F_2 H/L and FP Populations.

Genomic DNA was isolated using the Gentra Generation Capture Plate™ Kit from the whole blood according to the Capture Plate™ Kit protocol (Gentra Systems, Minneapolis, USA). In total, 208 microsatellite markers were chosen for both populations. For the analysis, only the informative markers for each population were chosen: 170 for H/L population and 180 microsatellite markers for FP population. The genotyping procedure was as already described by Siwek et al. [18, 19]. All genotypes were checked twice. Finally the data was checked for non-Mendelian inheritance using the CRI-Map [23]. All together, 718 animals from generations F_0 (28 individuals), F_1 (18 individuals), and F_2 (672 individuals) of H/L population, and 689 from generations F_0 (24 individuals), F_1 (35 individuals) and F_2 (630 individuals) of FP population were genotyped. In total, 208 microsatellite markers were tested on the H/L and FP grandparents for polymorphism. Thirty-eight markers for H/L and twenty-eight markers for FP could not be used in the total genome scan either because markers did not amplify, or the markers were not informative in the cross. The map distances in general did not differ much from the distances on the consensus linkage map therefore the map distances based on the consensus map were used. The estimated genome coverage is 78% for the H/L population and 80% for the FP population.

Genetic analysis

To estimate the heritability for LPS and LTA and their genetic correlations and genetic correlation with KLH, uni- and bi- variant analyses were performed using an animal model and the ASREML software package [24]. Since the level of NAbs could be dependent on the maternal genotype, preliminary analyses were performed with an additive/maternal

model. No indications were found for the existence of a maternal effect. For this analysis the following mixed model was used:

$$Y = X\beta + Z\mu + e$$

where Y is a vector of observations, X is the design matrix for the fixed effects, β is the vector of fixed effects, Z is the design matrix for random effects, u is the random effects with var (u) = $A\sigma_a^2$, and e is the residual with var (e) = $I\sigma_e^2$. The fixed effects for the H/L population are: hatch and sex, and hatch for the FP population.

QTL Analysis

The analyses were performed separately for both experimental populations.

Prior to the QTL analysis, phenotypic data were adjusted for the systematic: hatch (for FP population) or hatch and sex (for H/L population) effects using the PROC GLM Procedure [25].

For the FP population: $Y_{ij} = \mu + H_i + e_{ij}$

For the H/L population: $Y_{ijk} = \mu + H_i + S_j + e_{ijk}$

Where Y_{ij} or Y_{ijk} is the phenotypic value, μ is the grand mean, H_i is the effect of the i_{th} hatch (i = 1, 2,.... , 5 for FP population, and i = 1, 2,.... , 6 for the H/L population, respectively); S_j is the sex effect of jth individual; and e_{ij} or e_{ijk} represents residual effects. The hatch is referred to a group of individuals hatched at one time.

Regression interval mapping was used for QTL detection. Two different genetic models were used: 1) paternal half-sib analysis [26, 27] and 2) line-cross analysis model [28]. In the paternal half-sib model no assumptions are made concerning the allele frequencies in the founder lines and number of QTL alleles. The F_2 animals are treated as number of unrelated half-sib families using the model:

$$Y_{ij} = m_i + b_i p_{ij} + e_{ij}$$

where Y_{ij} is the trait score of individual j, originating from sire i; m_i is the average effect for half-sib family i; b_i is the substitution effect for a putative QTL; p_{ij} is the conditional probability for individual j of inheriting the first paternal allele, and e_{ij} is the residual effect.

In the line-cross model, the power of QTL detection depends on the degree of fixation of QTL alleles for the trait of interest in the founder lines. In this model the alternative alleles at the QTL are traced back to the founder lines. At every centi Morgan across the genome the following model is fitted:

$$Y_j = m + ax_{aj} + dx_{dj} + e_j$$

where Y_j is the adjusted trait score of animal j, m is the population mean, a and d are the estimated additive and dominant effect of a putative QTL at the given location, x_{aj} is the conditional probability of animal j carrying both alleles from the same line, x_{dj} is the conditional probability of being heterozygous at given location, and e_j is the residual error.

Significance Thresholds

Significance thresholds were determined empirically by a permutation test [29]. Data permutation, with at least 10,000 replicates, was used to determine the empirical distribution of the test statistic under the null hypothesis of no QTL associated with the chromosome under study [27]. Two significance levels were defined according to Lander and Krugylak [30] were 1) suggestive linkage (one false positive is expected in a genome scan); and 2) significant linkage (statistical evidence expected to occur 0.05 times in a genome scan).

Results

Phenotypic Measurements

H/L Population

The mean NAbs level $\pm$ SD for LPS at 5 wks of age was 3.52 $\pm$ 1.46. The mean level $\pm$ SD for LTA at 5 wks of age was 1.56 $\pm$ 1.23. The mean level $\pm$ SD for LPS and LTA at 18 wks of age was 7.96 $\pm$ 1.49 and 7.46 $\pm$ 1.44 respectively.

Heritability and genetic correlation between Nabs level to LTA, LPS and KLH (SpAb response) are presented in Table 1A. In general, the heritability for all the traits is rather low both at 5 and 18 wks of age. The standard error for the genetic parameters is high, due to the low number of animals available for genetic parameter estimation.

Table 1A. Heritabilities (on diagonal), and genetic (above diagonal) and phenotypic (below diagonal) correlation for LPS, LTA and KLH in H/L population . Standard error in parenthesis.

Variable	LPS 5 wks (SE)	LTA 5 wks (SE)	LPS 18 wks (SE)	LTA 18 wks (SE)	KLH (SE)
LPS 5 wks	0.17 (0.06)	0.55 (0.29)	0.07 (0.30)	-0.03 (0.31)	NE
LTA 5 wks	0.00	0.03 (0.03)	0.02 (0.63)	0.43 (0.31)	0.86 (0.40)
LPS 18 wks	0.16	0.04	0.09 (0.04)	0.02 (0.63)	0.13 (0.34)
LTA 18 wks	0.04	0.13	0.44	0.16 (0.06)	0.92 (0.11)
KLH	0.02	0.12	0.03	0.37	0.07 (0.03)

LPS – lipopolysaccharide, LTA - Lipoteichoic acid, KLH – keyhole lymphet hemocyanin

NE - not estimable

FP Population

The mean Abs level ± SD for LPS and LTA at 38 wks of age in the FP population was 6.37 ± 1.16 and 8.07 ± 1.08 respectively.

Heritability and genetic correlation are presented in Table 1B. Heritability of LPS and LTA in the FP population is 0.23 and 0.42, respectively. Genetic correlation is high (0.79) between LPS and KLH, 0.77 between LTA and KLH, and 0.78 between LPS and LTA.

Table 1B. Heritabilities (on diagonal), and genetic (above diagonal) and phenotypic (below diagonal) correlation for LPS, LTA and KLH in FP population . Standard error in parenthesis.

Variable	LPS (SE)	LTA (SE)	KLH (SE)
LPS 38 wks	0.23 (0.10)	0.78 (0.25)	0.79 (0.20)
LTA 38 wks	0.50	0.42 (0.14)	0.77 (0.16)
KLH	0.24	0.25	0.11 (0.07)

LPS – lipopolysaccharide, LTA - Lipoteichoic acid, KLH – keyhole lymphet hemocyanin

QTL analysis: paternal half-sib model

H/L Population

QTL detected with the half- sib analysis model are presented in Table 2.

For the antibody response to LPS at 5 wks of age a suggestive QTL was detected on GGA8 in marker bracket MCW0160 and ADL0345. For the antibody response to LPS at 18 wks of age a suggestive QTL was detected on GGA3 in marker bracket MCW0005 and ADL0144.

For the antibody response to LTA at 5 wks of age a suggestive QTL was detected on GGA3 in marker bracket MCW0038 and MCW0222. A suggestive QTL on GGA10 was detected for the antibody response to LTA at 18 wks of age in marker bracket ADL0209 and MCW0067.

FP Population

QTL detected with the half- sib analysis model are presented in Table 2.

Table 2. QTL for antibody responses to LPS and LTA using the half-sib analysis model.

Trait	Chromosome	Marker Bracket	Position (cM)	Test statistic	R^2
		H/L population[1]			
LPS 5 wks	GGA8	MCW0160–ADL0345	38	4.06*	0.04
LTA 5 wks	GGA3	MCW0038–MCW0222	66	3.09*	0.03
LPS 18 wks	GGA3	MCW0005-ADL0144	106	3.93*	0.04
LTA 18 wks	GGA10	ADL0209–MCW0067	56	3.15*	0.03
		FP population[2]			
LPS 38 wks	GGA8	MCW0160-ADL0345	42	2.54*	0.04
	GGA18	MCW0045-ROS0022	17	3.53*	0.05
LTA 38 wks	GGA3	MCW0126-MCW006	276	4.28**	0.05
	GGA14	MCW0296–ADL0118	1	2.92*	0.04

[1]H/L – high and low lines

[2]FP - feather pecking population

* denote suggestive linkage

** denote genome wise significance at 5% level.

LPS – lipopolysaccharide, LTA - Lipoteichoic acid

R^2 - phenotypic variance explained by QTL

Two suggestive QTL were detected for the antibody response to LPS at 38 wks of age. A first QTL was detected on GGA8 in marker bracket MCW0160 and ADL0345. A second QTL was detected on GGA18 in marker bracket MCW0045 and ROS0022.

Two QTL were detected for the antibody response to LTA at 38 wks of age. A significant QTL was detected on GGA3 in marker bracket MCW0156 and MCW006. A suggestive QTL was detected on GGA14 in marker bracket MCW0296 and ADL0118.

QTL analysis: line-cross model

H/L Population

QTL detected with the line – cross analysis model are presented in Table 3.

Two suggestive QTL were detected for antibody response to LPS at 5 wks of age. A suggestive QTL was detected on GGA4 with dominance effect (SE) of -14.89 (4.54) and additive effect (SE) of 0.29 (0.50). The second suggestive QTL was detected on GGA12 with dominance effect of -1.70 (0.42) and additive effect of -0.30 (0.31). Four QTL were detected for antibody response to LPS at 18 wks of age. A significant QTL was detected on GGA1 with dominance effect of -2.28 (0.55) and additive effect of 0.070 (0.31). Three suggestive QTL were detected. A first QTL was detected on GGA4 with dominance effect 14.94 (4.70) and additive effect 0.59 (0.40). The second QTL was detected on GGA7 with

dominance effect of 2.05 (0.66) and additive effect of 0.38 (0.23). The third QTL was detected on GGA18 with dominance effect of -1.82 (0.56) and additive effect of 0.23 (0.23).
For the antibody response to LTA at 5 wks of age, two suggestive QTL were detected. The first QTL was detected on GGA2 with dominance effect of -0.75 (0.22) and additive effect of 0.27 (0.14). A second QTL was detected on GGA3 with dominance effect of -1.20 (0.36) and additive effect of 0.51 (0.22).
A suggestive QTL was detected for antibody response to LTA at 18 wks of age on GGA2 with dominance effect of 1.28 (0.34) and additive effect of 0.29 (0.19).

FP Population

QTL detected with the line – cross analysis model are presented in Table 3.

Table 3. QTL affecting non specific immune response to LPS and LTA detected using the line-cross analysis model.

Trait	Chromosome	a (SE) [3]	d (SE) [3]	Marker Bracket	Position (cM)	Test statistics[4]	R^2
			H/L [1]				
LPS 5	GGA4	0.29	-14.89	MCW0180- MCW0174	194	5.61*	0.02
	GGA12	-0.30	-1.70 (0.42)	MCW0198-MCW0332	76	8.79*	0.03
LTA 5	GGA2	0.27	-0.75 (0.22)	ADL0197-MCW0042	193	7.77*	0.02
	GGA3	0.51	-1.20 (0.36)	MCW0059-MCW0252	184	7.83*	0.02
LPS 18	GGA1	0.07	-2.28 (0.55)	ADL0359-MCW0112	184	9.09**	0.03
	GGA4	0.59	14.94 (4.70)	MCW0180-MCW0174	207	6.43*	0.02
	GGA7	0.38	2.05 (0.66)	MCW0133-MCW0183	77	5.94*	0.02
	GGA18	0.23	-1.82 (0.56)	ROS0022-MCW0219	47	5.97*	0.02
LTA 18	GGA2	0.29	1.28 (0.34)	GCT0020-ADL0197	187	8.3*	0.03
			FP[2]				
LPS 38	GGA27	0.23	-0.44 (0.22)	MCW0300-MCW0076	25	4.21*	0.02
LTA38	GGA7	-0.24	1.16 (0.47)	MCW0133-MCW0183	78	3.83*	0.02
	GGA14	0.30	1.17 (0.35)	MCW0123-MCW0225	51	8.02**	0.04

[1]H/L – high low population

[2]FP – feather pecking population

[3]Estimated QTL effects for the genetic model. The (a) additive; the (d) is the dominance genes effect. Standard error in parenthesis.

[4]Test statistics against H_0 of no QTL

*denote suggestive linkage

LPS – lipopolysaccharide, LTA - Lipoteichoic acid, 5 wks, 18 wks and 38 wks of age.

R^2 – phenotypic variance explained by QTL

A suggestive QTL was detected on GGA27 for the antibody response to LPS at 38 wks of age with dominance effect of -0.44 (0.22) and additive effect of 0.23 (0.11).

For the antibody response to LTA at 38 wks of age two QTL were detected. A significant QTL was detected on GGA14 with dominance effect of 1.16 (0.35) and additive effect of 0.30 (0.14). A suggestive QTL was detected on GGA7 with dominance effect of 1.16 (0.47) and additive effect -0.24 (0.16).

Discussion

In the current study results are presented of an experiment dealing with the level of Natural antibodies measured by homotopes LPS and LTA in two independent populations: H/L and FP cross.

The NAb level against LPS and LTA for the H/L population increased between 5 and 18 wks. Moreover, the level of LTA responses in the H/L cross at 18 wks of age is almost the same as the level of LTA responses in the FP population at 38 wks of age. The mean LPS level at 38 wks of age in the FP population is even lower than in the H/L population at 18 wks of age. It has to be noticed that before 5 wks of age the experimental H/L population didn't encounter any specific antigens, except for the standard immunization used in poultry keeping, whereas by the age of 18 wks immunized with a number of antigens, e.g. KLH, *M. butyricum*, SRBC and *E.coli*. In contrary the FP population was not immunized till the age of 36 wks, when the individuals were injected with the KLH. This may suggest that the overall level of NAbs rises more effectively in H/L population being stimulated by specific antigens at regular intervals starting at young age (5wks) [18, 19]. In contrast to the FP population in which immunisation started only at 36 wks of age hence the NAb level showed the natural incresement with age of the individual [31].

These findings are in agreement with the observations that the level of NAbs increases with age of the individual, which corresponds with the secretion of Abs by naturally occurring auto – reactive B cell clones (FP population) or reflects polyclonal stimulation by exogenous microbes initiating responses of auto-reactive B cells (H/L population) [31].

Both populations described in this study were specifically set up for QTL mapping of traits other than Nabs. In this study the pedigree structure is not optimal for the estimation of genetic parameters due to the limited number of animals. This will result in a large standard error on the estimates. Nevertheless, the results presented here give an indication that there is a genetic effect on the regulation of Nabs.

QTL for level of natural antibodies were detected. In general, the majority of detected QTL are different . Firstly, the QTL are different for the two homotopes LPS and LTA which means the known different nature of LTA and LPS. The suggested different immunomodulatory features of LPS and LTA in opposite directions were already confirmed [32] in the founder lines of current H/L population. In addition, in mammals it has been shown that TLR's play an important role in the regulation of the immune system. Although

TLR's have not been fully identified in non mammalian species, it has been shown that LTA and LPS bind to different TLR's e.g., LTA is binding TLR-2 and LPS is binding TLR-4.

Secondly, QTL identified for both homotopes differ at both ages. The exception is a QTL on GGA2 for LTA level which is shared at 5 wks and 18 wks of age what this suggests that some genes related to the immune response/ immune regulation at both ages are located on GGA2.

Thirdly, QTL detected for level of Nabs differ from previously detected QTL for SpAbs. In general chromosomal positions of the QTL detected for LPS and LTA (non-specific) immune response differ from those detected for (specific) antibody response to SRBC [18], KLH, and *M.butyricum* [19]. In brief, major regions associated with primary antibody responses to SRBC in H/L population are located on GGA1, and GGA16, whereas QTL for primary antibody responses to KLH is detected on GGA3.

In the current analysis two statistical models were used: the half – sib and the line cross analysis model. The highest number of QTL was detected under the line cross analysis model in H/L population. Generally it is believed that line-cross analysis is more powerful to detect QTL then the half-sib analysis. However, if the alleles deviate from fixation the power drops very fast [33]. Because both methods revealed different QTL it is not likely that QTL alleles for Nabs are fixed in the founder populations. Parmentier [11] has reported higher Nab response in the H line then in the L line. These lines were specifically selected for Ab response to SRBC. The genetic correlations between SRBC and Nabs in the H/L population are near zero (data not shown). The H line also shows higher response to KLH then the L line. All this, indicates that selection for specific Ab can influence the level of Nab titers. Even though there is no one to one relation between Nab level and specific Ab response, there may be an increase in QTL alleles for Nabs as well.

Of particular interest is the QTL detected on GGA14. This QTL was identified for specific antibody response to KLH in H/L population, and was later confirmed in the independently created FP cross. The same region also shows significant linkage with LTA response under the half-sib and the line – cross analysis model in FP cross, suggesting a common genetic mechanism between NAb and specific Ab response. The reason for non detection of the QTL for LTA response in the H/L population might be the time difference in KLH administration before LTA level measurement in both populations which was 2 wks for the FP cross and 6 wks for the H/L population. This is in line with the study of Lammers et al. [12] suggesting a functional relation between Nab level and specific antibody responses. In addition, Parmentier [32] observed that LPS administration decreases the

antibody titers to KLH, LTA and to LPS itself, whereas LTA and KLH enhance antibody responses to LTA, KLH and LPS. This indicates that the prior former antigen administration (KLH) does influence the measured level of NAbs against homotopes. However, the phenotypic variance explained by the QTL for KLH [19] and LTA is very small. Therefore it is difficult to predict whether the same gene(s) are involved or not. Further analysis using multi-trait analysis assuming relation between the traits might give an improvement of the power to detect the QTL as well as a better estimation of the QTL positions [34].

The detected QTL regions are too large to enable identification of positional candidate genes. A first step in decreasing these regions will be incorporating additional markers in the region of interest by using for example Single Nucleotide Polymorphism Markers (SNP). Subsequently after narrowing down the QTL regions, comparative mapping will be used, based on the information from human/ chicken and mouse/chicken comparative genetic map. Especially since the complete DNA-sequence of the chicken will be available in the very near future is expected to be a powerful tool for the identification of positional candidate genes. So far, GGA14 is not well characterized and it is difficult to come up with possible candidate genes. Identification of the avian counterparts would be an important step in understanding the regulation of innate immunity in chickens. A second region of future interest is GGA3, where QTL for specific Ab response to KLH as well as QTL for Nabs level were detected. On GGA3 several genes known to have immunomodulatory features are located (i.e.TGFB, TLR5)

In summary, the results of a QTL study aiming at identification of regions associated with (nonspecific) immune responses to LPS and LTA in two independent populations of laying hens is presented. In general different QTL regions were detected for both homotopes (LPS and LTA), except for the QTL on GGA14, which indicates association with non specific responses to LTA, and examined earlier, responses to KLH. In addition, association of different QTL regions detected for non specific immune responses and different QTL regions detected for specific immune responses is suggested.

This study on the genetic architecture of NAbs in chicken has provided evidence that the NAb level in chicken has a genetic component and different QTL were detected. There is a high genetic correlation found between NAbs and specific Ab response to KLH in the FP population, however, further fine-mapping is necessary to verify whether the same genes are involved in the QTL region on GGA14.

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CHAPTER 5

Fine mapping of QTL for primary antibody responses to KLH in laying hens.

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Introduction

A total genome scan has been applied to identify quantitative trait loci (QTL) in an experimental population that was created from High (H) and Low (L) selection lines, where the H and L lines were divergently selected for primary immune response to Sheep Red Blood Cells (SRBC). A QTL for KLH (keyhole limphet hemocyanin) was detected on GGA14 (Siwek et al, 2003). KLH is a novel antigen for birds and, therefore, a suitable antigen to study antibody (Ab) response as part of the Immune Response. The QTL for primary antibody response to KLH on GGA14, was also detected in an unrelated population created from commercial chicken lines which showed different behavioural features in relation to feather pecking (Buitenhuis et al., 2003). The detection of the QTL in two independent populations justifies further analysis in terms of fine mapping and progressed inter crossing (F_3 and F_4).

In the whole genome scan approach microsatellite markers were used to identify QTL. However, when all available microsatellite markers were used, the identified region on GGA14 still spanned over 50cM. Similar confidence intervals for identified QTL were reported in other studies (e.g. de Koning et al., 1999; Boichard et al., 2003). When working with model organisms or plants, strategies to improve the mapping resolution most often involve breeding a large number of progeny to increase the density of crossovers in the chromosomal region of interest (Darvasi *et al.*, 1993). When working with humans or farm animals, this approach is not practical, and alternative strategies need to be identified. One approach that has recently received considerable attention is linkage disequilibrium (LD) mapping, which aims at exploiting historical recombinants.

A promising approach in fine mapping a QTL region is combining information obtained by linkage analysis (LA) and linkage disequilibrium analysis (LD) (Meuwissen and Goddard, 2000). In the LA approach information transmitted from the parents to their offspring is traced with DNA markers. If LA (roughly within family information) is combined with LD (roughly between family information), additional knowledge about historic recombination events is utilized. Closely linked markers provide more accurate information to the linkage analysis. This type of approach was successfully applied to fine map QTL in dairy cattle (Meuwissen et al., 2002; Olsen et al., 2004). To the authors' knowledge, presented study gives first evidence for successful fine mapping with combined LDLA approach in poultry.

The LD or LDLA approach requires an increase in marker density as useful LD is expected to extend only over limited distances. This implies that additional markers are

needed for fine-mapping a QTL in order to point out positional candidate genes. SNP markers are far more abundant than microsatellites; in chicken a SNP frequency of 1 SNP per 100bp is observed (Vignal et al., 2000, ICGSC, Nature 2004). This SNP abundance makes it very suitable for haplotype analysis.

The current study focused on fine mapping of a QTL for primary antibody response to KLH: (1) through the addition of SNP's (2) through the implementation of novel analysis methods to maximise use of information across F_0–F_4 generations and (2) through a continued inter cross All of these steps should facilitate identification of possible candidate gene for primary antibody response.

Material and methods

Chicken Population.

The H/L F_2 population originated from a cross (ISA Warren, medium heavy layers) between two divergently selected lines for either high (H line) or low (L line) primary antibody response to SRBC. Selection was based on the individual antibody titre at 5 d after primary intramuscular immunisation with SRBC at 37 d of age (Van der Zijpp and Nieuwland, 1986). Reciprocal crosses with birds from the 18th generation were made to generate F_1 animals. From the F_1 generation, an inter-cross was made to produce 672 individuals in 6 hatches of the F_2 experimental population. For the QTL detected on GGA14 in the F_2 generation, using within family regression, only one family showed the significant founding for QTL effect. For this reason, this family was used to produce generation F_3 and subsequently generation F_4 was created. F_4 generation consisted of 3 half-sib families with an average of 100 offspring per sire.

All birds were housed in brooder cages with free access to water and feed (152 g/kg CP and 2,817 kcal/kg ME). Birds were not beak-trimmed and each individual bird was marked with a wing-band. All birds were vaccinated against Marek's disease, infectious bronchitis, and infectious bursal disease at hatch, and at 2 and 15 d of age, respectively. The Ethical Committee On Animal Care and Welfare of Wageningen University, The Netherlands approved the experiment.

Phenotyping of the generations F_2 and F_4.

Total antibody responses to KLH were measured in individual plasma samples obtained at 7 d after s.c. immunization with 1 mg KLH (Cal Biochem-Novabiochem Co., La Jolla, CA) in 1 mL PBS (pH 7.2) at 12 wk of age. Antibody titers to KLH of all birds were measured by

an indirect ELISA as described by Sijben et al. (2000), and as performed in the previous experiment (Siwek et al., 2003). Titers were expressed as the $\log_2$ values of the highest dilution giving a positive reaction.

SNP detection

Animals

The SNP discovery panel consisted of eight animals. All of them originated from one F_1 family which had the highest contribution to QTL effect. In the panel: F_1 sire, two F_1 dams, and 5 F_2 individuals, ancestors of F_4 generation were present.

Primer design

In total 58 STSs were tested for amplification and sequencing. Primers were designed from end sequences of BAC clones that contained loci mapped on GGA14 or based on the Beijing SNP database (Wong et al., in press). All primers were designed using Primer3 through the web interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

PCR and sequencing

PCR reactions were carried out in 24µl volumes. Reactions were performed as described by Jungerius et al. (2003). After PCR reaction the excess of primers was removed by running the samples over a column of BioRad P-100 (BioRad, Hercules CA , USA) in a Multiscreen MAHV N45 plate (Millipore, Billerica, MA, USA) in 96-well plate format. From each sample, 2µl was checked on agarose gel to estimate the DNA concentration. Sequencing reactions were performed with the forward and the reverse amplification primer. The sequencing reactions contained 100 – 400 ng of purified PCR product, 2µl of Big Dye Terminator Rtmix (Perkin – Elmer, Foster City, CA , USA), 2µl of Half Big Dye Buffer (Genetix, New Milton, UK) and 0.8 pmol of either primer in a final volume of 10µl. Excess dye terminator was removed by running the samples over a column of Sephadex G-50 (Amersham Pharmacia, Uppsala, Sweden) in Multiscreen MAHV N45 plate format. Subsequently, the samples were dried using a Speed Vac and analysed with DT3100POP6 module, 96 well plate on an ABI3100 sequencer.

SNP identification

Sequencing gel images were analysed using the Sequencing Analysis Software (ABI) for lane tracking and trace file extraction. All trace files were analysed with the Pregap4 program of the Staden software package (Bonfield & Staden, 1996; http://www.mrc-lmb.cam.ac.uk/pubseq).

Genotyping strategy of the F_2 H/L population.

Genomic DNA was isolated using the Gentra Generation Capture Plate™ Kit from the whole blood according to the Capture Plate™ Kit protocol (Gentra Systems, ver. 5.00, Minneapolis, MN, USA). The genotyping strategy of the QTL region consisted of three steps:

Step 1 (M1). microsatellite markers used in the whole genome scan approach, genotyped on the entire F_2 population (6 half – sib families) (Figure 1).

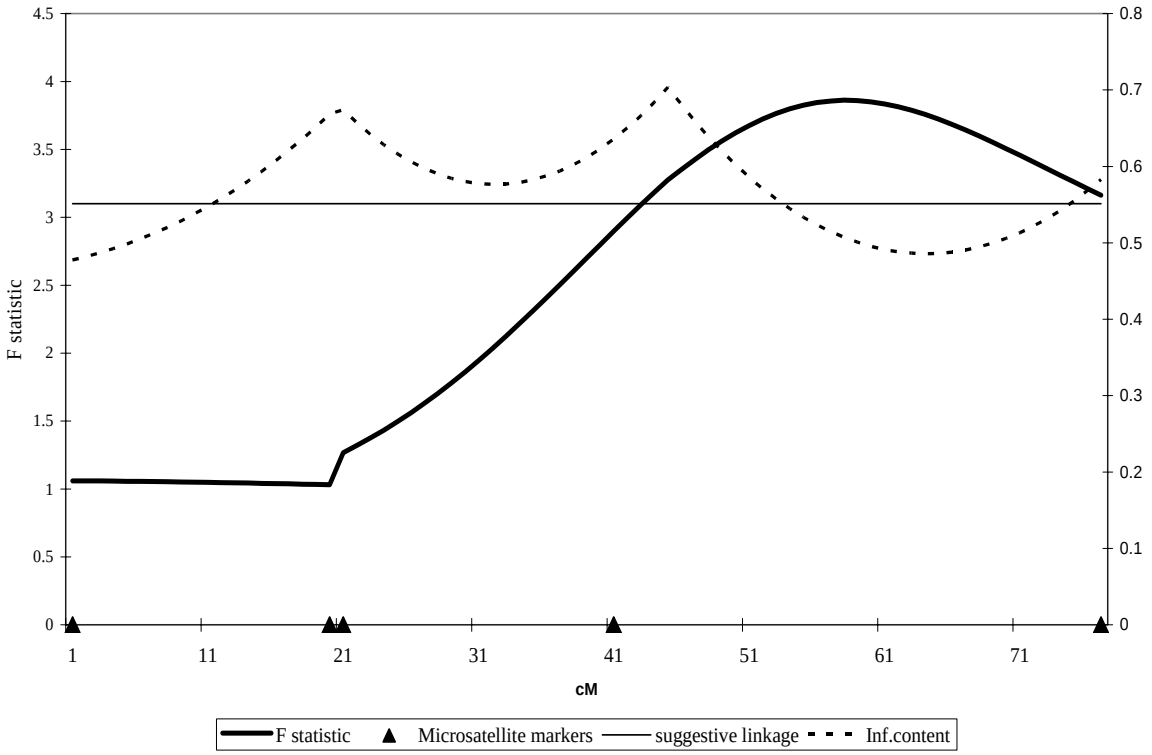

Figure 1. Regression interval mapping of primary antibody response to KLH on GGA14. Analysis of the entire F2 population.

Step 2 (M2) First set of SNP markers was genotyped on one F_2 half–sib family (i.e. the one that contributed the most to the QTL detected on GGA14). Based on this family, SNP

markers were selected that were within the desired marker brackets and that were most informative based on marker variance component analysis (data from one half – sib family). Step 3 (M3) pre-selected markers (step 2) were combined with additional, new SNP markers in one set of 16 SBE primers and used to genotype the entire F_2 population (6 half – sib families). The markers are summarised in Table 1. This marker information is used in LDLA analysis.

Table 1. Microsatellite markers and SNP markers, located on GGA14 used in LDLA analysis of F_2 generation of H/L population.

Marker name	Contig on GGA14	Marker position ** (cM)
MCW0296	Ctg 64015	6
MCW0136	Ctg 64015	20
ADL0118	Ctg 64015	21
MCW0123	Ctg 1301	45
SCW0296	Ctg 1301	46
SCW0290	Ctg 1301	47
SCW0293	Ctg 1301	49
SCW0292	Ctg 1301	61
SCW0298	Ctg 1301	52
SCW0283	Ctg 1301	53
SCW0326*	Contig 383.12	55
SCW0318*	Contig 48.48	58
SCW0319*	Contig 48.117	59
SCW0320*	Contig 48.144	70
SCW0324*	Contig 48.16	72
SCW0323*	Contig 192.5	74
SCW0325*	Contig 192.44	75
MCW0225		76
SCW0273	Ctg 63806	77

* denotes SNP defined based on Benjing data base.

** as used in regression linkage analysis

Step 1 was described by Siwek et al. (2003). For step 2, preliminary QTL analyses with (1) a paternal half – sib model, and (2) marker variance component model, were performed. SNP markers with made a significant contribution to the QTL variance were identified. Seven out of 16 SNP markers contributed to the over all QTL effect. In total 9 additional SNP markers were developed and included in the final set for step 3 (16 SBE primers).

SNP Analysis with SnaPshot kit

To generate the template for the SNP analysis, PCR amplification was performed in a total volume of 12 μl with multiple primers. The exact volume of primers was defined experimentally for each combination. PCR reactions were performed with Accuprime ©. Each reaction contained: 5 μl of DNA of a final concentration 10 ng / μl, 1 μl of ddH2O, 10 μl Accuprime, 4 μl of primer mix of final concentration 200 nM. The Accuprime PCR amplification program was: 1 cycle of 2 min at 94C, 40 cycles of 30 sec at 94C, 30 sec at annealing temperature (50C, 55C or 60C), 3 min at 68C, followed by 1 cycle of 2 min at 68C. Subsequently the PCR products were checked on an agarose gel. After the amplification PCR products were pooled up to the final volume of 15 μl. In the next step, all PCR products were cleaned from primers and dNTPs with SAP/ExoI treatment. Before the Snapshot reaction all primers were premixed to a concentration of 0.2 μM for each primer. The Snapshot reaction was performed in a total volume of 10 μl consisted of: 3 μl of pooled and treated PCR products, 2 μl of pooled SnaPshot primers, 4 μl of Half Big Dye buffer and 1 μl of SnaPshot Ready Reaction Mix. The program of PCR was: 1 cycle of 5 min at 96C, 40 cycles of 10 sec at 96C, 5 sec at annealing temperature, 30 sec at 60C. After the extension, all samples were treated with SAP enzyme (1 unit / 1 μl) for 1h at 37C, followed by 15 min at 72C to inactivate the enzyme. The sequencing reaction was performed on ABI 3100 (Applied Biosystems).

QTL Analysis using Regression Interval Mapping.

Prior to the QTL paternal linkage regression analysis, phenotypic data were adjusted for the systematic hatch (H_i) and sex (S_j) effects using the PROC GLM Procedure (SAS Institute, 1995).

$$Y_{ijk} = \mu + H_i + S_j + e_{ijk} \qquad (a)$$

Where μ is the grand mean, H_i is the effect of the i_{th} hatch (i = 1, 2,...., 6), S_j is the gender effect and e_{ijk} represents residual effects. The hatch is referred to as a group of individuals hatched at one time.

Regression interval mapping was used for QTL detection. A paternal half-sib analysis model (1) was applied (Knott et al., 1996; De Koning et al., 1999). In the paternal half-sib model no assumptions were made concerning the allele frequencies in the founder lines and number of QTL alleles. The F_2 animals were treated as unrelated half-sib families using the statistical model:

$$Y_{ij} = m_i + b_i P_{ij} + e_{ij} \qquad (b)$$

where Y_{ij} is the trait score of individual j, originating from sire i; m_i is the average effect for half-sib family i; b_i is the substitution effect for a putative QTL; P_{ij} is the conditional probability for individual j of inheriting the first paternal gamete, and e_{ij} is the residual effect.

QTL analysis using Marker Variance Component Analysis.

As an approach in step 2, variance component analysis (2) was applied, where model (a) was extended with the F_2 individual as a random animal effect, including three generations of the pedigree. This model was continued with the effect of Mp – marker allele inherited from sire side and Mm marker allele inherited from dam side:

$$Y_{ijk} = \mu + H_i + S_j + a_l + Mp_m + Mm_n + e_{ijklmn} \qquad (c)$$

with *a*, *Mp* and *Mm* as random effects

The procedure tested if addition of marker information (each marker separately) improved the likelihood of the model. Derivation of haplotypes, that is determine whether a specific marker allele is of paternal or maternal origin, was performed with SimWalk2 (Sobel & Lange, 1996). Variance component calculations were performed using ASReml software package (Gilmur, 2000).

QTL analysis using combined Linkage and Linkage Disequilibrium (LDLA .

Meuwissen and Goddard (2001) developed a multimarker linkage disequilibrium and Linkage analysis mapping method was developed for the fine mapping of quantitative trait loci (QTL) using a dense marker map. The method compares the expected covariances between haplotype effects given a postulated QTL position to the covariances that are found

in the data. The expected covariances between the haplotype effects are proportional to the probability that the QTL position is identical by descent (IBD) given the marker haplotype information. The joined data of microsatellite- and SNP markers (SNP set 2) was analysed in the combined Linkage Disequilibrium and Linkage (LDLA) Analysis approach. Haplotypes were derived using SimWalk2 (Sobel & Lange, 1996). The phenotypic records of F_2 animals were analysed using the statistical model:

$$Y = Xb + Zh + e \qquad (d)$$

Where *Y* is the vector of records, *b* is the vector of fixed effects (sex, batch), *h* is the vector of random effects of the haplotypes, *e* is the vector of residuals; *X* and Z are known incidence matrices for the effects in *b* and *h* respectively.

The program was also used in for a situation in which LD information was ignored, i.e. linkage analysis (LA) only, for comparison with the regression method analysis.

Two genetic inheritance models were tested in the LDLA analysis: one single additive genetic variance component (1 variance, combined sire and dam component) and a separate sire and dam genetic variance component (2 variances). The 2-variances model was tested against the 1- variance model in order to detect parent-of-origin effects. In the larger marker brackets (more than 11cM) the QTL was fitted at several positions within the interval. In smaller marker brackets, the QTL position was assumed to be in the middle of the bracket.

Candidate genes approach.

The Twin Scan (http://genes.cs.wustl.edu/) software package for homology gene prediction was applied to DNA genomic sequence from the QTL region. 9 possible candidate genes were detected in the region of interested, based on the sequence homology. Subsequently, obtained protein sequences were blast (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The profile of sequences comparison was as followed: matrix BLOSUM62, blastp, significance threshold e = 0.01.

Results

Best position for the QTL

1. Results of paternal half-sib regression analysis (One half-sib family)

In Figure 2 the results of the regression approach are given for the original microsatellites (M1), microsatellites plus first set of SNP's (M2) and the final analysis with the extra set of

SNP's added (M3). Adding more markers (M2, M3) decreased the confidence interval but also decrease the significance of the detected QTL. The highest test statistic equalled 18.2 for the microsattelite markers (M1), 10.9 for analysis with the first set of SNP markers (M2), and 13.7 for analysis with the final set of SNP markers. The a-posteriori positions of the second group of SNP's (M3), based on observed recombination fractions was different from the design based on the draft map. The 9 SNP's were chosen to be at less than 1 cM distance from one another, but turned out to be dispersed over the distal part of GGA14. Three SNP's could not be placed on GGA14 due to very high recombination rate, suggesting positions on other chromosomes.

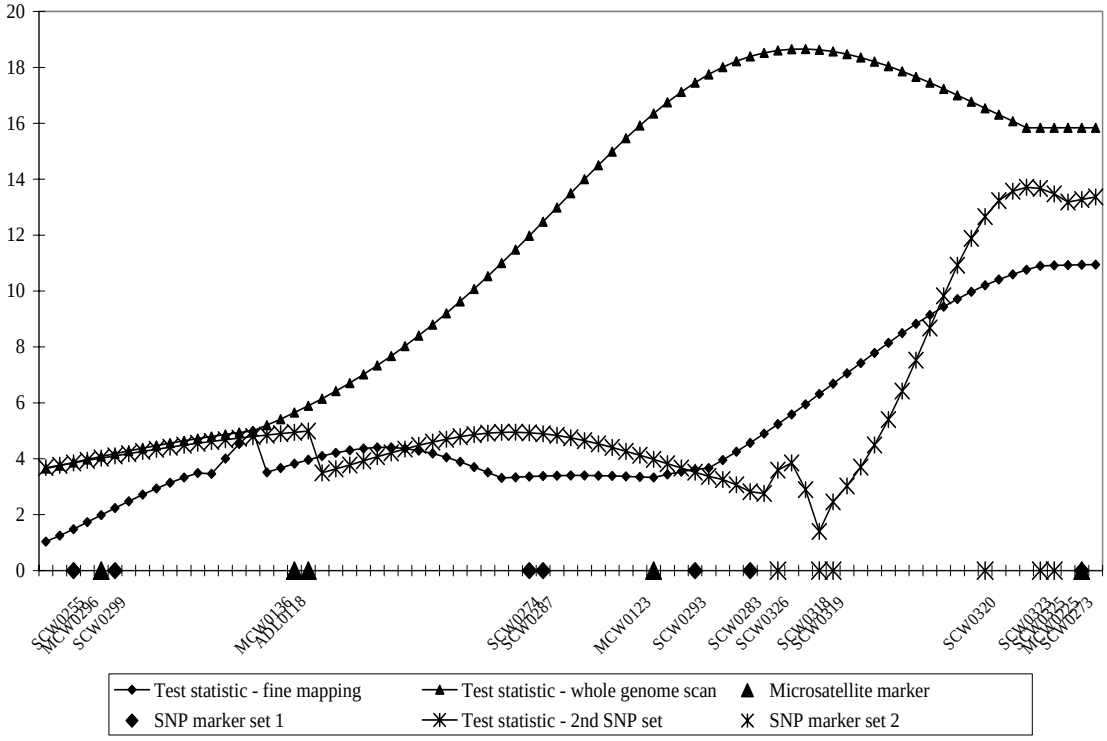

Figure 2. Regression interval mapping of primary antibody response to KLH on GGA14. Analysis of one family.

2. Results of LDLA analysis of entire F_2 population.

The entire F_2 population was genotyped for 5 microsatellite markers (M1) and 16 SNP markers (Table 1). The analysis was performed in several steps. First the LA 1 variance and LA 2 variances were tested against the null hypothesis. In the next step the combined analysis LDLA was performed, for both LDLA 1 variance and LDLA 2 variances model. The LDLA 2 variances model was tested against LDLA 1 variance to check for imprinting.

In Figure 3A results are given for the LA analysis and Figure 3B for the combined LDLA analysis. In both analyses, the highest test statistic was found around position 76 at the distal end of GGA14. The maximum test statistic for the LA 2 variances analysis was 6.39 (p = 0.05) and for the LDLA 2 variances analysis was 7.69 (p = 0.005). Significance thresholds derived from the log-likelihood ratio test indicated that both approaches found QTL affecting KLH response. The QTL was narrowed down to the region below 1 cM. The QTL peak falls in the marker bracket: SCW0325 and MCW0225. The estimated distance between these two markers is 0.1cM. The LDLA analysis with the 2-variance component model explained the data better than the single variance model, the LR test equal to 7.69.

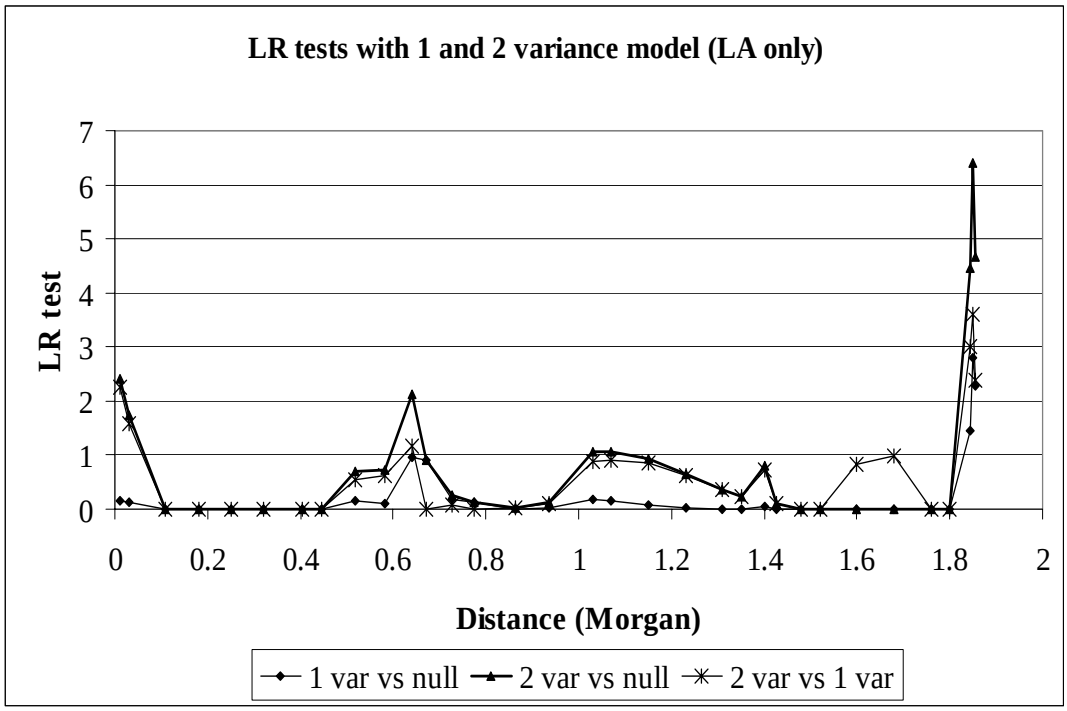

Figure 3A. Linkage analysis of primary antibody response to KLH on GGA14.

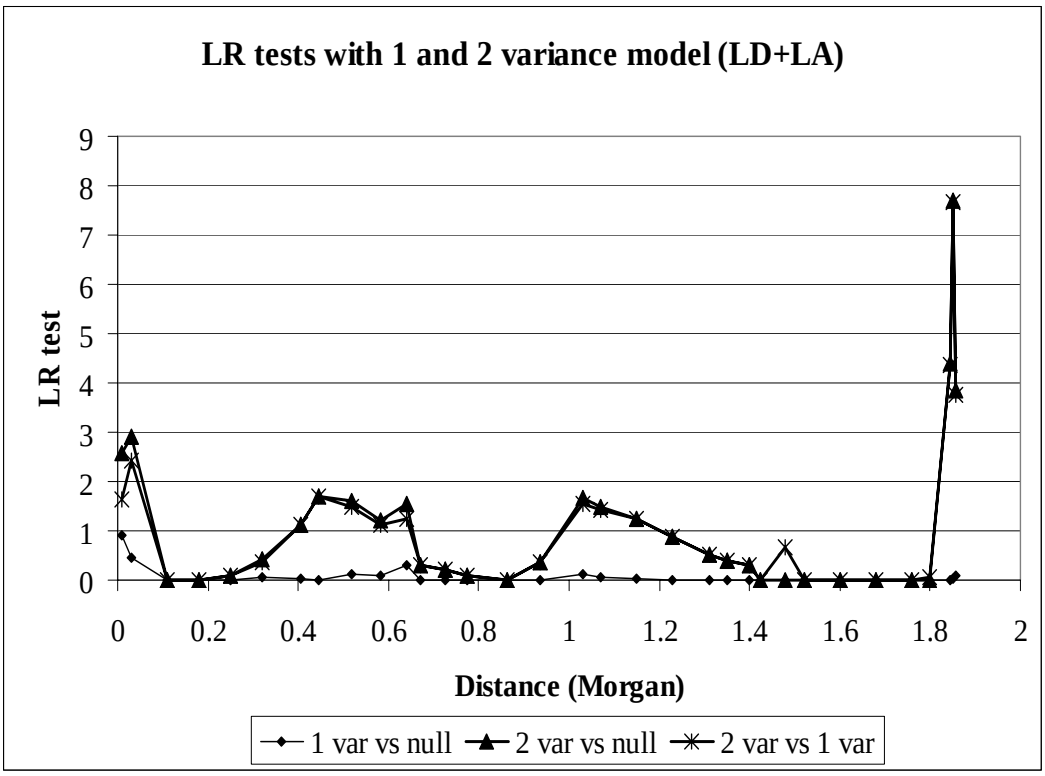

Figure 3B. Combined Linkage and Linkage Disequilibrium analysis of primary antibody response to KLH on GGA14.

3. Size of the effect.

The allele substitution effect estimated in the regression linkage analysis with 5 microsatellite markers was 1.34 (Siwek et al. 2003), what means 0.7 genetic standard deviation. The allele substitution effect estimated in the linkage analysis with 5 microsatellite markers and first set of SNP markers reduced to 0.77. For the final set of 5 microsatellite markers and second set of SNP markers the estimate effect was 0.71, what means 0.53 genetic standard deviation. Estimates of haplotype effects were in the order of 0.6 for the high alleles, indicating a QTL of 1.0 to 1.5 genetic standard deviations.

Heritability estimated in the LDLA analysis of the F_2 population was 0.12. This implies that 12% of the phenotypic variance in KLH response can be attributed to additive genetic effects unlinked to the QTL on GGA14.

The variance components for the LDLA analysis are presented in Table 2.

Table 2. The variance components for LDLA analysis.

Components	LA analysis		LDLA analysis	
	1 variance	2 variances	1 variance	2 variances
δ^2_a	0.159	0.006	0.307	0.09
$\delta^2_{QTL\ paternal + maternal}$	0.052	-	0.0028	-
$\delta^2_{QTL\ paternal}$	-	0.20	-	0.22
$\delta^2_{QTL\ maternal}$	-	0.00	-	0.005
δ^2_e	2.37	2.45	2.33	2.40

4. Effect of segregating haplotypes in F_2 generation.

Based on LDLA results, the most significant contribution was defined and consists of last four markers. Three sires share the same (2 – 2 – 181 – 1) haplotype related with high KLH effect. The high KLH effect was in range from 0.52 to 0.59. In the other group, the low KLH effect clear haplotype grouping could not be done. The estimates of hapolotype effects are presented in Table 3.

Table 3. Effect of segregating sire haplotypes in F_2 generation.

Sire	Haplotype				Effect
428	1	2	181	3	-0.2904
471	2	4	187	3	-0.2852
409	2	4	187	3	-0.1797
327	2	4	187	3	-0.1796
471	2	4	187	3	-0.1561
327	1	2	181	3	-0.1178
448	2	2	177	3	-0.0688
302	2	4	187	3	-0.0098
448	1	2	181	1	0.1285
428	2	2	181	1	0.5139
302	2	2	181	1	0.5724
409	2	2	181	1	0.5951

5. Results of paternal half – sib regression analysis of F_4 generation.

The regression interval mapping analysis of the F_4 data didn't reveal presence of QTL effect for primary antibody responses on GGA14. The allele frequencies, for MCW022 marker linked with the QTL effect in F_2 generation 5 was 0.37 (allele 181) and 0.63 (allele 187).

6. Results of candidate genes.

The candidate gene approach pointed out five genes, two chicken genes annotated in QTL region and three human homologues. The chicken genes are: noggin precursor (AC 093525) and Netrin 2 precursor (AC Q90923). The human homologues are: serine/ threonine protein kinase (AC Q8IV63), G2 mitotic specific cyclin F (AC P41002) and TBC1 domain family member (AC Q9BXI6).

Discussion

In order to be able to identify genes underlying quantitative traits, several steps have to be taken. First the QTL region has to be detected, subsequently validated in an independent population and finally fine mapped. This line of study was undertaken here and a QTL on GGA14 was followed from detection with a whole genome scan in the H/L population, through validation in an independent population and to a fine mapping step in the original H/L population. With regard to the QTL for primary antibody response to KLH three findings from current study will be discussed: the position of the QTL, the probable imprinted nature of this QTL and possible candidate genes.

Fine mapping

Siwek et al. (2002) reported a QTL for primary antibody response to KLH on GGA14 at position 3 cM, in the marker bracket MCW0296 and MCW0123. This analysis unfortunately used an incorrect marker order. Re-analysis of the data (reference) revealed the best position of the QTL at microsatellite MCW0225 at 77 cM (reference paper corrected by erratum). To narrow down the QTL region additional SNP markers were added to the data set. Additional marker information narrowed down the QTL peak, but also decreased the test statistic. Despite the additional markers, confidence interval was still too big to be able to come up with positional candidate genes in QTL region. Based on the marker variance component information for each of the SNP markers, the most suitable set of SNP markers was created, and based on the Benjing SNP data base information additional SNP's were detected. Marker variance component approach evaluated the potential relevance of a particular SNP and decreased the number of SNP necessary for further analysis. This type of approach was suggested by Shifman and Darvasi (2004) in their simulation study on mouse inbred strain. The most informative markers in term of QTL effect were: MCW0225, and two neighbouring SNP, one before the MCW0225 marker (SCW0325) and the other at the end of the chromosome (SCW0273). No recombination between SCW0325 and MCW0225 were found. The original approach dealing with the data set was based at classical linkage analysis. In current study, linkage disequilibrium was added to investigate whether or not between historic information on LD could be used to map this QTL more precise. Results presented in Figure 3B, indicate that overall significance is only slightly higher than in the situation with LA only (Figure 3A). Explanation of this observation could be fact that the interesting QTL allele segregates only in one family at the paternal side. There is no

contribution to the overall QTL effect in other families, therefore combine information is limited.

The application of LDLA approach to narrow down the QTL was very successful. The QTL detected with microsatellite markers and paternal half – sib regression analysis spanned over 50cM. The fine mapping data and LDLA analysis narrowed down QTL region below 1cM.

Parent-of-origin effect

Until now little evidence for the existence of parent-of-origin in chickens were reported (Tuiskula- Haavisto et al., 2004, Siwek et al., 2003). In the current study, an additive QTL model was tested against the null hypothesis and significant confirmation for the alternative hypothesis was found. Subsequently separated sire/dam variance model was tested against the additive model and again it gave a significant improvement. Excluding the dam effect did not result is a loss of significance. Therefore the interesting marker allele (MCW0225) in term of QTL effect was followed, through the pedigree to the F_1 sire and the F_0 grandsire. The specific allele was present in two F_1 individuals, one sire and a full sib dam and was inherited from low SRBC F_0 sire. Divergent selection to SRBC of High and Low lines (ancestors of the current experimental populations), influenced KLH response. The High SRBC line had higher KLH antibody titres compared to Low SRBC line, which responded lower to KLH. The F_1 sire was founder of the half – sib family which contributed the most to the overall QTL effect on GGA14. Although, the F_1 dam had 50 offspring and segregated the MCW0225 allele still this full sib family didn't show the QTL effect. As follow up, from the interesting sire (409) F_2 next generations a F_3 and F_4 were created. The lack of the QTL effect in F_4 generation could be explained by parent-of-origin haplotype segregation. Despite of the proper allele frequencies of the MCW0225 in the F_4 population the expected QTL effect was not present. Unfortunately, the interesting MCW0225 allele (181) was inherited in F_4 animals only through F_3 dams and not through F_3 sires. At present, the parent-of-origin effect (imprinting) is still under discussion in oviparous species such as birds. In the current results statistical evidence for a parent of origin effect is supported by a negative (lack of effect) proof. The actual biological evidence for parent-of-origin effect in poultry should come from expression studies at the RNA and protein level.

Candidate genes.

Despite all the detail information available for the QTL region on GGA14 in term of DNA sequence, and very small marker bracket, performed candidate gene detection was not successful. It has to be keep in mind that the annotation in the distant part of the chicken chromosomes is not very accurate as well as comparative map with human genome is not very detailed. Several genes were predicted; nevertheless none of them seem to be a good candidate for KLH antibody response.

In summary, the results of QTL fine mapping are presented. The study suggests novel analysis approach to evaluate the potential relevance of particular SNP to the dissected trait. Successful application of relevant SNP markers and combined LDLA analysis allowed us significantly narrow down QTL region. The haplotype block related with high KLH effect was defined and uncovered in the F_1 sires.

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CHAPTER 6

General Discussion

Despite intensification of sanitation and constant improvement of vaccination and medical care, disease outbreaks in populations of food animals including poultry still occur. Increasing domestic livestock production conditions and the strong increase in animal density favors the spread of infectious diseases. To reduce the spread of diseases and to increase animal welfare, opportunities to improve disease resistance of animals are considered important. Selection for overall enhanced disease resistance, for example, is an interesting approach to improve animal populations. To implement a selection scheme for improved disease resistance, a more complete knowledge of its genetic basis is needed. To that end, High and Low chicken selection lines were initiated at Wageningen University. In these lines, the immune response towards a non-pathogenic multi antigen – SRBC – was measured and used as a criterion for selection over several generations. The initial idea of this experiment was to enhance the general immune responsiveness and improve overall disease resistance. Selection for a broad multi repertoire response, to such as SRBC might accomplish this goal. (van der Zijpp and Nieuwland, 1986). These selection lines (High responders, Low responders and Control line) have been intensively studied across generations. In generation 10, High and Low lines differed in various immune characteristics. With regard to the T cell dependent antigens (KLH, TNP, and BSA) the High line had higher antibody responses compared with the Low line. There was no difference between lines in responses to T cell independed antigens (*Brucella abortus*, *Salmonella*) and a macrophage activity. T cell activity in vitro was higher in the Low line than in the High line. With respect to MHC haplotypes, the High line was almost homozygous for the MHC haplotype B-^{21}, and the Low line was homozygous for B-^{14}. Based on literature on other species, the MHC was expected to be a main component determining immune responses. However, the earlier work of Pinard (1992) demonstrated that variation in the MHC complex within and between the selection lines was not the only factor explaining genetic variance in disease resistance and immune competence. The current thesis is a follow up of the research on the Wageningen selection lines, with a special attention to the analysis of the genetic basis of specific immune responses at the molecular level. The purpose of the research described here, is to address following questions:

1. Are genes involved in immune responses different (or similar) for different antigens? If yes, selection for these genes could add to selection for general enhanced resistance.
2. Is it possible to describe functional relationships among various elements of immune responses?
3. Is it possible to use information of QTL detected for immune response traits to improve health? E.g. by applying this to breeding programs.

To address the question about genetic control of the specificity of immune responses, apart from the SRBC (selection criterion), a broad range of antigens was used. Among them were common bacterial antigens: *E. coli* and *M. butyricum*; in addition an antigens to which birds were never exposed: KLH, and ConA were used.

The results for adaptive (humoral) immune responses to several antigens are described in chapter 2, 3 and 5, and results for the innate (natural) immune response are in chapter 4.

1. Adaptive immunity

The chicken adaptive immune system involves humoral as well as cell-mediated responses. As is true for mammals, cell mediated immunity is regulated by CD3+ T cells probably via the release of different cytokines that results in either: the Th1 or Th2 pathway. The Th1 (inflammatory) response is stimulated by TNF, IFNγ and related cellular responses. The Th2 pathway is probably similar to mammals being stimulated by IL4, IL5, and IL10 and results in a humoral response (Erf, 2004).

Is has been proposed that chickens do not elicit typical Th2 responses, and that the chicken immune system lacks components which are under the regulation of the Th2 cytokines as in mammals. For these reason the chicken immune system may require less cytokines from the Th2 “class”. Indeed, it has been shown that the chicken orthologue of the gene for the Th2 cytokine, IL-5,is a pseudogene (Avery et al. 2004). On the other hand, the IL4 and IL13 were recently discovered in birds (Smith et al. 2004), both representing Th2 class cytokines in mammals.

Significant line differences were found with respect to antibody titers to KLH (Th2) and *M. butyricum* (Th1) (Parmentier et al., 1998) in the founder lines of the current H/L population. Antibody responses to both antigens were always higher in the H line. Sijben et al. (2001) demonstrated differences in the H/L selection lines between the antibody responses to KLH and *M. butyricum* indicating that kinetics and modulation were different.

1.1. Relations among antigens

The antigens, used in this experiment, might be group as follows in Table 1.

Table 1. Antigens used in current experiment are classified in two categories based on their requirement for a T cell compartment.

Antigen	T cell dependent	T cell independent
SRBC primary	+	
SRBC secondary	+	
KLH	+	
E.coli	+	
M. butyricum	+	
LPS		+
LTA		+

Group 1 consists of T cell dependent multi determinant (complex) antigens represented by: KLH, *M. butyricum, E. coli* and SRBC for which primary responses were determined. In addition, SRBC secondary response was also measured. Group 2 consisted of the T cell independent antigens represented by: LTA, LPS to which natural antibodies were determined. With regard to the cellular (Th1) vs. the humoral (Th2) pathways, majority of the antigens (SRBC, KLH, *E.coli*) represent Th2 type of responses in mammals. The Th1 type of response in mammals is represented by *M.butyricum*. Taken all of the above into account, common locations of several genes that underlie the regulation of specific immune responses were expected for the group of the T cell dependent antigens. Within this group common QTL should be detected for SRBC primary and /or, SRBC secondary response, and/or SRBC antibody responses and/ or KLH antibody responses. All these three antigens are T cell dependent ones; all of them follow the Th2 response in mammals. With respect to *M. butyricum*, a genetic region related with regulation of immune response to this antigen might be different from regions related with immune response to KLH and SRBC. Although both antigens are T cell dependent ones, each one follows different T cell response (Th1 or Th2) in mammals. Genetic regions related with innate immune responses to the T cell independent antigens (LTA and LPS) were expected to be different from the T cell dependent antigens.

1.2. Genetic parameters

Estimation of the heritability was used as the first step in unraveling the genetic background of traits. The heritability measures the amount of additive genetic variation within a population as proportion of total phenotypic variance. The estimated heritabilities of the traits in this study ranged from 0.07 for KLH to 0.16 for LTA and SRBC primary response (Chapter 4). This result is in agreement with the literature (Martin et al., 1990; Siegel Gross 1980; Bovenhuis et al., 2002). The estimated heritabilities indicate that genetic factors contribute to the variation between animals. However it does not provide information on a common background of different immune traits nor does it provide information on the number or the location of the genes

Genetic correlations between immune responses for different antigens were estimated to determine they were partially under the same genetic control. Estimated genetic correlations between the antigens used in this study are presented in Table 2.

Table 2. Genetic correlations, SE are in parenthesis.

	SRBC primary	SRBC secondary	KLH	LPS 5 wks	E. coli	LTA 5 wks
SRBC primary	--	0.45 (0.35)	-0.39 (0.34)	NE	NE	NE
SRBC secondary	0.45 (0.35)	--	0.29 (0.39)	NE	NE	NE
KLH	-0.39 (0.34)	0.11 (0.39)	--	NE	NE	NE
E. coli	0.74 (0.29)	0.51 (0.38)	0.38 (0.38)	NE	NE	NE
M.butyricum	0.23 (0.39)	0.73 (0.22)	0.65 (0.25)	NE	0.66 (0.24)	NE
Con A	0.16 (0.28)	0.15 (0.23)	0.31 (0.22)	0.40 (0.34)	NE	0.46 (0.36)
LPS 5 wks	0	0.03 (0.39)	0.77 (0.21)	NE	NE	NE
LTA 5 wks	0	0	0.83 (0.29)	NE	NE	NE
LPS 18 wks	0.05 (0.34)	0.17 (0.39)	0.01 (0.42)	NE	NE	NE
LTA 18 wks	0.13 (0.42)	0.51 (0.24)	0.85 (0.19)	NE	NE	NE

NE – not estimated

Genetic correlations were high for some of the antigens studied, e.g. SRBC primary and *E. coli* (0.74) and KLH and LTA 5 wks (0.83). This reveals that there is a substantial overlap in genes affecting these traits. A genetic correlation of 0.8 indicates that both traits have 64% of the genetic variance in common. For others traits the genetic correlations were moderate to low, suggesting that there is little overlap in the genetic mechanism underlying the variation in these traits. Only for one combination (KLH primary response and SRBC primary response), a negative genetic correlation was found. This occurs when an increase

in genetic value for one trait is associated with a decrease for the other. The fact that most correlations are positive, support the thesis of a regulatory mechanism common to these traits.

A genetic correlation between traits can be caused by close linkage of genes or by pleiotropic effects. For traits that show a high genetic correlation, it is expected that QTL that having an effect on one trait would also affect the other. Some overlapping QTL regions were expected between antigens being highly correlated (large than 0.7). The SRBC primary response was expected to share QTL regions related with primary antibody responses to *E. coli*.

We found a moderately high genetic correlation (0.65) between the antibody responses to KLH and *M. butyricum.* Given the nature of both antigens, being Th1-like and Th2-like low or no genetic correlation was expected. A possible explanation might be that the time between KLH and *M.butyricum* injections was too short (2 wks) and the true *M. butyricum* effect might have been masked by the previous KLH effect. In other words, immune response once pushed towards Th2 (KLH) type of response, needs more time to regain "neutral" Th0 status. Recently it was shown that immunization with KLH affected the immunocompetence still 6 -7 weeks later (Parmentier , personal communication).

With respect to relations between T cell dependent and T cell independent antigens common QTL region were expected for SRBC secondary response and LTA levels at 18 wks of age. Thus, also a QTL for antibody response to KLH was supposed to share the QTL location with LTA levels at 5 wks of age and with LTA level at 18 wks of age. The homotope LTA has been suggested to enhance Th2 antibody responses. Indeed LTA when given to poultry stimulated antibody responses to KLH. In contrast, QTL for LPS might not be related to the region underlying Th2 like responses. LPS, as a Th1 antigen in mammals affected antibody responses in a negative way.

1.3. QTL results of adaptive immune response

The QTL described in this thesis concern several antigens: *M. butyricum,* KLH (Chapter 3), SRBC (Chapter 2), *E. coli* and ConA. The overall picture of detected QTL is presented in Table 3.

Table 3. QTL detected with the whole genome scan approach.

Chromosome	Location (cM)	Trait	Model	Immune response	Positional candidate gene
GGA1	184	LPS 5 wks	LC**	Innate	
	395	SRBC primary	HS*	Adaptive	Interleukin 1 Receptor-Like 1
GGA2	27	E coli	LC*	Adaptive	
	190	LTA 18 wks	LC*	Innate	
	190	LTA 5 wks	LC*	Innate	
	325	SRBC primary	LC**	Adaptive	
GGA3	20	KLH	HS*	Adaptive	
	70	LTA 5 wks	HS*	Innate	
	70	Con A	LC*	T cell activation	
	106	LPS 5 wks	HS*	Innate	
	125	KLH	LC**	Adaptive	
	184	LTA 5 wks	LC*	Innate	
	283	E coli	HS*	Adaptive	
	310	SRBC primary	LC**	Adaptive	Interleukin 17A
GGA4	84	M. butyricum	HS*	Adaptive	
	84	E coli	LC*	Adaptive	
	106	SRBC primary	LC*	Adaptive	Interleukin 15 precursor
	194	LPS 5 wks	LC*	Innate	
	207	LPS 18 wks	LC*	Innate	
GGA6	20	E coli	LC*	Adaptive	
GGA7	77	LPS 18 wks	LC*	Innate	
	105	SRBC primary	LC*	Adaptive	
GGA8	38	LPS 5 wks	HS*	Innate	
GGA10	56	LTA 18 wks	HS*	Innate	
GGA12	78	LPS 5 wks	LC*	Innate	
	78	Con A	LC*	T cell activation	
GGA13	1	KLH	LC*	Adaptive	Interleukin 12B
	54	SRBC primary	LC*	Adaptive	
GGA14	60	KLH	HS*	Adaptive	
	60	KLH	LC*	Adaptive	
GGA16	3	SRBC primary	HS*	Adaptive	
GGA18	45	SRBC secondary	LC*	Adaptive	
	45	LPS 18 wks	LC*	Innate	

*denotes linkage at genome suggestive level
** denotes linkage at genome significant level
HS = half sib analysis model

LC = line cross analysis model

KLH is an antigen that birds do not encounter during their lifetime, and thus represents a novel antigen, suitable to measure primary immune responses. KLH is a complex antigen just as SRBC. Responses to such complex antigens are rarely exclusively Th1 or Th2 mediated. The combination and dominance of certain Th cytokines will drive the response to such an antigen complex toward cellular or humoral mediated pathway (Jankovic et al., 2001; Gause et al., 2003). QTL detected for SRBC primary and secondary responses (Chapter 2) are located on different chromosomes than QTL to the two other complex antigens (*M. butyricum*, and KLH). This might indicate that even if the immune response to an antigen follows the same pathway, there is additional complexity in the control of responses to different antigens. An interesting explanation would be that unknown genes playing a role in the control of immune reactivity could be detected using this approach. Alternatively, common QTL (suggested by the genetic correlation) were not detected given the power of this experiment.

If one summarized the QTL results described in this thesis, (Chapter 3) for immune responses to KLH (Th2) and *M.butyricum* (Th1), no overlapping QTL regions for these antigens were detected. The reason could be the insufficient power of the experiment, therefore only QTL with very big effects were detected. However, another explanation might be the already mentioned, skewed immune response towards a Th2 pathway caused by the KLH injection. The interval between KLH and *M. butyricum* injection was too short, this might explain the lower numbers of QTL detected under both models (half-sib and line cross), for *M. butyricum* (Th1-like) responses.

A common location for QTL for different antigens was not observed for the GGA16, region where the MHC is located. The Major Histocompatibility Complex (MHC) plays a crucial role in the control of specific immune response. MHC molecules fulfill the role of recognition of every protein antigen. There are three classes of MHC gene products: class I (expressed on the surface of all the cells), class II (expressed on B cells, APC and stimulated T cells) and also in chickens class IV - expressed on the surface of erythrocytes. Especially chicken MHC, among major histocompatibility complexes in other species is of a special interest. The chicken MHC is the minimal essential set of genes, and this poultry an interesting model to study relations of MHC with immune responses. Indeed, strong relations between MHC type and level of disease resistance have only been found in chickens (Kaufman 1996).

The presentation of antigen in the form of antigen – peptide bound to self-MHC class II molecules allows CD4+ T cells that have TCR specific for the antigen – peptide – MHC class II complex to engage their TCR with this complex. Yonash et al. (2000) suggested an association of MHC class IV with antibody responses to SRBC, *E.coli* and NDV based on RFLP markers information. However, it should be noted that MHC structure in general are very complex, and highly polymorphic. It was already indicated, that MHC is not the only player in the immune responses (Pinard, 1992). In the previous study concerning the present parental selection lines approximately 3.5% of the total variation of interline differences was attributed to MHC. The molecular marker information available in this study was limited. In the whole genome scan approach two microsatellite markers (all available) mapped on GGA16 were used to obtain molecular data. Therefore, given the limited power of the present experiment, the association of MHC with immune response to KLH or *M. butyricum* cannot be excluded. It should be indicated that in the High and Low selection lines only two (B14 like and B21 like) MHC haplotypes were present. Furthermore, given the complexity of the antigens used it is unlikely that SRBC, *M. butyricum* or *E. coli* were not bound by MHC haplotype B14 or B21. A QTL confounded in the MHC region might have been found when simple peptides (e.g. GAT) would have been used (Chen and Lamont 1992). QTL for immune response to KLH associated with GGA16 was found in FP population (described in more detail in Chapter 3). This FP population was selected for production traits and therefore, might be expected that a higher number of MHC haplotype was present in this resource population.

The genes that underlie adaptive immune responses are scattered across the entire genome. Different QTL regions are linked with various antigens. We did not find a single region in the genome that explains a large part of the specific humoral immune response. Thus selection for improvement of overall immune responsiveness based on a single QTL seems not feasible. In selection schemes, information on a larger number of QTL needs to be used.

2. Innate immunity

In the next part of this thesis, attention was focus towards the innate immune system. The question asked was to find genetic components of innate immune responses and relations between the innate and adaptive immune system could be found.

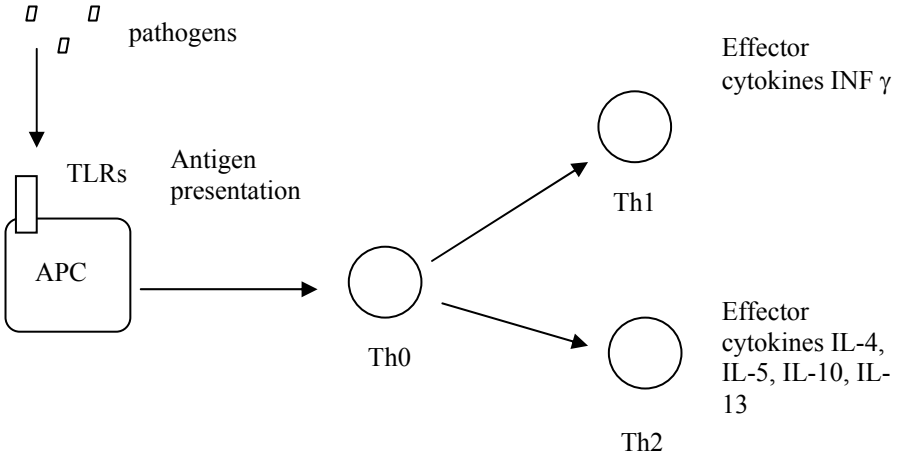
Initially, the immune response towards any antigen is in the hands of the innate immune system. This is a subject of intensive research in man and mouse after being

discarded for a couple of decades. Several complementary mechanisms are at play in innate immune responses, although the finesses (rules) are not fully understood at present. Nevertheless, it is clear that several cell types, e.g. phagocytes or dendritic cells and natural killer cells, but also acute phase proteins, complement, and the recently acknowledged natural antibodies play a role in the innate immune response. Upon exposure to pathogens, one of several paths can follow: infection might lead to death or chronic disease, might be terminated by innate immunity itself or might be taken over by adaptive immunity. The role of innate immunity is emphatic. Invertebrates survive infections with an innate immune system only. In addition, mutant mice with no adaptive immunity can survive and reproduce as long as they don't have contacts with the non mutant ones (Parham, 2003).

Natural Antibodies (Nabs) are probably the first reactive molecules in the whole cascade of immune responses to encounter foreign antigens. Although their action is not fully understood, it has been shown that they are involved in antigen presentation to T cells by e.g opsonisation, activation of Antigen Presenting Cells (APC). All organisms recognize invading antigen pathogens by Pathogen-Associated Molecular Patterns (PAMPs) shared by most pathogens. At the side of the host, PAMPs are recognised by pattern recognition receptors (PRR). PRR are expressed on cells that encounter the pathogen in the first place i.e.: APC, macrophages, epithelial cells. PRR represent three classes of receptors: 1. endocytic, 2.-secreted proteins, and 3. signaling receptors. Nabs can be attributed to the second group. The third class is represented by Toll Like Receptors (TLR) and additional signal proteins like TRAIL, MyD88 (TLRs use the four adapters: MyD88, Mal, Trif, and Tram in different combinations).

Binding to Toll proteins and TLRs activate many immune response genes. Recently at least 10 TLRs genes have been identified in human. Among them TLR4 has been shown as a recognition molecule for LPS, and TLR2 – a recognition molecule for LTA. Of the ten human TLRs reported to date, two TLRs were cloned from chicken bursa cDNA, and designated and chTLR type 1 and 2. Both of them show the highest homology to human TLR2. Both genes were mapped to chromosome 4q1.1, suggesting that they arose by gene duplication (Fukui et al., 2001). Furthermore, TLR 1/6, TLR2 type 1, TLR2 type 2, TLR3, TLR4, TLR5 and TLR7 were reported as expressed by chicken heterophils (Kogut, 2000). Regulation of Th response through TLR and links between innate and adaptive immune responses is presented in Figure 1.

Figure 1. Relation between innate and adaptive immunity.



2.1. QTL in innate immune responses

Innate immune recognition is based on pathogen – associated molecular pattern (PAMPs). Two examples of PAMPs used in this study are: LPS and LTA. LPS and LTA represent components of bacterial cell walls shared by Gram-negative bacteria, and Gram-positive bacteria, respectively. These two homotopes (LPS, LTA) were used to measure the level of natural antibodies (Nabs) which have recently been re-acknowledged as an integral part of the innate immunity system binding PAMP.

With regard to Nabs, it is shown that they not only exist in chickens but also we found a genetic component related to Nabs level (Chapter 5). LTA as an indicator of naïve immune humoral reactivity had a heritability of 0.16, almost identical to the heritability of SRBC primary response. The low genetic correlations between Nabs level to LTA and LPS indicate that the genetic regulation of innate humoral immune responses is different at different ages (5wks and 18 wks) and different for both investigated homotopes. This is supported by the lack of QTL that influence both traits.

QTL detected for SRBC primary response (SpAb) and Nab level to LPS and LTA do not point to the same regions. This observation is in agreement with estimated genetic

correlations between SpAb to SRBC primary response and Nabs levels to LPS and LTA, which is low (0 to 0.15 depending on age). Parmentier et al. (2004) showed that selection for high specific antibody (SpAb) (SRBC) response lead to a correlated increase in level of Nabs. This suggests that common genetic factors contribute to both traits. The Nabs levels were measured at different ages in both experiments, and might account for some of the observed differences. The latest measurement in the H/L population was at 18 wks of age, whereas in selection lines the Nabs level was estimated at 1.5yr of age. The level of Nabs rise with the age of the individual, therefore it might be suggested that there is an age dependency of the functional relation between SpAb (SRBC primary response) and Nab levels to LPS and LTA.

The QTL location presented in Table 3 is assumed to have a confidence interval of 20cM. Therefore, some common QTL locations might be pointed out. Interesting seems to be GGA2, where two QTL for LTA level are located, for early (5 wks) and late (18 wks) levels of natural antibodies to LTA suggesting the location of genes related to the immune response/ immune regulation at both ages. Two QTL were detected for two traits: LTA 5 wks of age, and Con A response both on GGA3. On the same chromosome, two other common locations were detected, one for LPS 5 wks of age and KLH and another for *E. coli* and SRBC primary response. Another two QTL were detected, one for *M. butyricum* and the other for *E. coli* on GGA4. Subsequently a common location of two QTL for two traits: Con A and LPS 5 wks of age is placed on GGA12. A common location in terms of statistical models (HS and LC) for QTL for primary antibody response to KLH GGA14 is observed. Two QTL were detected for two traits: SRBC secondary response and LPS 18 wks of age on GGA18.

On the way to determine common genes for different immune traits, at first heritability was estimated to qualify the inheritance part of the trait. Subsequently genetic correlations were estimated. For highly (> 0.7) genetically correlated traits some common QTL were expected. This is reflected by common QTL location for KLH and LPS 5 wks (GGA3); and *E coli* and SRBC primary response (GGA3). However if the correlation is lower than 1, different genes may play a role as well. For lower correlated traits smaller overlap in term of the QTL location was expected, although a common QTL location for lower correlated traits are observed as well e.g. SRBC secondary response and LPS 18 wks (GGA12). Our present results show that various locations contain genetic information for various immune responses to various antigens. Puel (et. al 1996) identified at least 10

genomic regions to be involved with the control of antibody response to SRBC only in an F2 cross of mice similarly selected for antibody response as the current chicken lines. Selection for the Biozzi mice was continued for much more than 18 generations as in chicken lines. In chicken lines, the current paternal Low line does not add much to further divergent selection. Different antigens will probably lead to different processing by innate and subsequently specific immune responses. Further responses are affected by the experimental design e.g. the age of the bird and route of administration of antigens.

3. QTL detection and beyond

Identification of the molecular genetic basis underlying important traits in all livestock species follows the same scenario. An appropriate resource population has to be designed and produced. The phenotypic and molecular genotypic data has to be collected and finally statistical analysis has to be performed (Lamont, 2003; Van Arendonk and Bovenhuis, 2003). In general, two types of resource populations might be taken into account: (1) crosses of inbred or selection lines and (2) segregating populations. The use of inbred lines or selection lines increase the power of QTL detection with the same number of animals as compared to non - inbred lines. It will reveal QTL that contribute to differences between the lines. An analysis of a segregating population will reveal QTL that contribute to genetic variation within a population. Both approaches have advantages and disadvantages (e.g. Van Arendonk and Bovenhuis, 2003). Fully inbred lines are rarely available for livestock species. Crosses between lines with extreme phenotypes are used instead. QTL experiments in poultry make use of layer - layer cross (Tuiskula - Haavisto et al., 2002), broiler - broiler cross (van Kaam et al., 1999), a broiler - layer cross (Sewalem et al., 2002). Additionally, crosses of unrelated lines (Schutz et al., 2002) or inbred lines (Zhou et al., 2001) are reported. QTL experiments in poultry dealing with immune traits include studies: Marek Disease Virus (Vallejo et al. 1998) and antibody response to *S. enteritidis* vaccine (Kaiser et al. 2002), and antibody response to *E.coli* vaccine (Yonash et al. 2001).

The advantage of the QTL approaches in detecting genetic basis is that there is no bias with regard to (preferred) genes related with investigated traits. As such, a QTL study can be viewed as an unbiased study. Therefore, any region, which is highlighted, is an independent confirmation of a gene or genomic region identified in other studies or expected on the basis of physiology.

The power of QTL detection depends on the size of the effect explained by QTL, heritability of the trait, population structure and size of the experimental population. With

regard to the size of the H/L populations (6 sires with 110 individuals per each sire), the heritability of analyzed traits (0.07 – 0.17), the estimated power was 0.88 for a magnitude of an QTL effect equal to 0.70, however the power dropped to 0.36 for QTL effect equal to 0.38.

Two models are used in data analysis in our study: half-sib (HS) and line cross (LC). In the half-sib (HS) model, QTL alleles are traced back to the sire, ancestor of the HS family. This analysis reveals QTL that are segregating with the population (heterozygous sires). In the LC analysis, QTL alleles are traced back to the selection lines and the analysis reveals QTL that contribute to differences between the lines. The LC analysis will only be able to detect a QTL when alleles are fixed (or close to fixation) in both lines.

The experimental design described in this thesis, allowed the use of both analyses. The lines used in this thesis were divergently selected for 18 generations for the primary antibody responses to SRBC. The LC is a more powerful model compared with HS when QTL alleles are fixed in the founder lines. A higher number of QTL was detected with the LC model (compared to HS).

It should be noticed that, QTL mapping experiments do not map genes but effects, which might consist of expression of many linked genes located even 30cM apart. Loci operating in opposite direction might thus not be detected. (Flint and Mott, 2001).

The most proper to dissect of complex traits is to start with QTL detection, and subsequently validate the QTL in a next generation and/or independent population. Only confirmed QTL justify the future fine mapping combined with candidate gene approaches. Such a line was followed in detection of QTL for KLH responses (chapter 3). This QTL was first detected on GGA14 in the H/L population; later this QTL was confirmed in an independent population. The F2 family of the H/L population, with the largest QTL effect was subsequently used to create generation F3 and F4 (chapter 5) which is the next step in reducing the confidence interval. The disadvantage of this approach might be that if QTL effect consists of a multiple gene effect, the Linkage Disequilibrium (LD) might break apart in further generations and the new QTL effect is too small to detect (Darvasi and Soller, 1995). To improve the resolution, and to narrow down the QTL region and define LD blocks a higher marker density is demanded. A QTL for the KLH antibody response on GGA14 was detected and validated using microsatellite markers mapped on this chromosome. However, even when all microsatellite markers available in this region at that time were used in the whole genome scan approach, the marker density was still too low (average

15cM apart). The markers of choice, only recently available, are single nucleotide polymorphism markers (SNP) which are more abundant through out the genome than microsatellite markers. In chicken even, the SNP frequency as 1SNP per 100bp is observed (Vignal et al., 2000). SNP markers can be used in association studies, however it is more efficient way is to combine SNP alleles and define SNP haplotypes. After discovery of haplotype blocks in human genome, a new idea was created about LD and haplotype diversity which can be captured by much smaller subset of haplotype tagging SNP (htSNP) (Johnson et al. 2001). htSNP is a subset of SNP that carry all or the most information found by the full set of SNP detected in the sample population. Studies applying htSNP or tagSNP mainly focus on human (Sebastiani, et al., 2003; Zhang et al., 2004; Carlson et al., 2004). Horne and Camp (2004) recently described an interesting follow up of this method. These authors proposed to introduce the concept of group – tagging SNP selected as SNP set that captures the desire proportion of the total genetic diversity.

4. Candidate genes.

The principal challenge with multifactorial traits is not in detecting the QTL, but to unravel the genes that underlie them. One possibility is to define positional and functional candidate genes by using comparative mapping (Schmid et al., 2000). The species of choice are human and mice because of their well-developed gene maps. Nevertheless, comparative mapping is viable alternative in chicken. However, it has been observed that there are multiple intra- and extra- chromosomal rearrangements between chicken – human and mouse (Crooijmans et al., 2001; Jennen et al., 2002; Jennen et al., 2003; Smith et al., 2002). To be able to identify positional candidate genes, a very detailed comparative map has to be available. To obtain a high-density comparative map many more genes must be placed. This might be done by using the information provided by chicken radiation hybrid panel (Morisson et al., 2002). Using radiation hybrid panel, a number of genes located on HSA 16p13 and HSA 17p11 were mapped to GGA14.

A number of chicken immune – related genes were identified in the large-scale EST project (Smith et al., 2004). Twenty-one different embryonic and adult tissues were used to create 64 cDNA libraries. Adding this EST collection to dbEST (http://www.ncbi.nlm.nih.gov) increases the number of chicken EST to 460, 000 and places chicken as the organism with the sixth largest EST collection. In a further step, the key is to predict which genes variants are likely to be functional *in silico*.To do this it is necessary to

identify every gene, protein product, promoter, transcription factor binding site, of the candidate region. Gene expression profiling seems to be an effective way to find candidates although its success depends on the nature of the phenotype. However, some obstacles remain. First QTL might produce only modest effects, which cannot be detected in microarrays at present. Second, there might be difficulties in identifying the proper tissue and development stage, complicating the choice of time point for sampling. Third, the mRNA differences might be secondary consequences of genetic action (Flint and Mott, 2001). Proper experimental design is a key issue for micro-array analysis of gene expression. The proper tissue has to be taken, from the proper individuals, at the proper moment of gene expression to be able to point to the candidate gene within the QTL region. In the current population there are only two families (out of 12) segregating QTL alleles for primary antibody response to KLH on GGA14. Even within the same population, the expression profile of the gene could be very different among families.

A promising approach for fine mapping QTL is to combine linkage analysis with linkage disequilibrium (LDLA) (chapter 6) to determine the minimum haplotype that is shared identical by descent (IBD). The IBD approach was successfully used in dairy cattle to identify DGAT2 and GHR and in pigs to identify IGF2 as causative genes for QTL effects. LDLA approach was applied to analyze fine mapping data for antibody response to KLH on GGA14. This narrowed the QTL region to the marker bracket of 0.1 cM. Based on LDLA result the most significant haplotype was defined, and consisted four markers. This haplotype was recognized in three sires, and linked with high KLH effect.

Positional candidate genes based on map information and functional gene predisposition (Kaiser et al.2004) like involvement in the regulation of immune responses may be suggested (Table 1). Some of the interleukins: IL–17A, IL–15 precursor, IL–1 RL1 are located within the QTL regions related with primary response to SRBC. IL– 12B is located within the QTL region linked with the KLH effect. All the positional candidate genes (cytokines) are located in the regions related with the adaptive immune response, whereas they are known as being involved in the innate immune responses. Therefore, it might be suggested a functional link between innate and adaptive immune responses. The information of the chicken genome sequence can be used in finding position candidate genes by applying gene prediction software (http://genes.cs.wustl.edu/; http://genome.imim.es/software/sgp2/) and comparing two genomic sequences. Such an approach was undertaken in detecting positional candidate genes for fine mapped QTL

detected on GGA14 for primary antibody response to KLH. None of the predicted genes seemed to be a suitable candidate related with primary antibody response to KLH. Nevertheless, QTL region of interest is located at the end of the chromosome, where the annotation is less precise.

5. Proteomics and immunoproteomics.

Since a draft version of the chicken genome is available (http://www.ncbi.nlm.nih.gov/genome/guide/chicken/), the chicken becomes the first livestock species that enters the new era. The sequence information speeds up genomics work. SNP could be defined directly from the sequence (Beijng SNP database). However, it has to be kept in mind that the current version is still a draft-version and not all the annotations are very precise. Even if the coverage is 6.6 times the haploid genome, there are still some gaps and mistakes in alignment possible. The estimated current chicken assembly covers 97 – 98% and it decreases and becomes more fragmentary in regions higher in GC content and representing extremes in repetitive content (International Chicken Genome Sequencing Consortium). The number of genes related with immune responses, known and annotated in chicken is not high (http://www.ensemble.org). DNA provides the coding information for all the biological process but the functional units of almost all biological processes are proteins. Protein expression very often has no correlation with mRNA expression. The estimated number of genes in human genome is ~20,000 but proteins produced by the human genome is 500,000, meaning that one gene alone produce more then 1,000 proteins. This complexity is provided by alternate mRNA splicing, which is followed by posttranslational modifications (Burgess, 2004). Immunoproteomics will provide valuable insights in disease pathogenesis and immunity by defining immune system function and dysfunction, pathogens, molecular systems in host immunity and pathogen immune evasion (Burgess, 2004). Le Naour et al. (2001) profiled changes in the transcriptome and the proteome during differentiation and maturation of monocyte- derived dendritic cells using DNA microarrays and proteomics. There was little concordance between cDNA and proteomics results. This suggests that to be able to get the complete picture how the system works transcriptomics and proteomics research have to be combined.

6. Selection for immune responses in relation to practice

Consumers demand animal derived products with the lowest participation of medical treatment and antibiotics as possible. Given the increased awareness in society regarding

animal welfare (Liinamo and Neeteson, 2003), disease resistance regained additional attention. In the coming years, important changes are expected in the European Union with respect to the system of egg production. Keeping hens in cages will be banned which has important consequences for the environment of the hen and the demands on her immune system. In the future floor system, contact with manure and higher dust and ammonia levels are important risk factor affecting health of animals.

Disease resistance may be divided into resistance to infections and resistance to disease development (disease tolerance) (Axford & Bishop 2002). Complete resistance to one pathogen is rare and usually inherited through one gene. Disease tolerance is less specific with regard to pathogen type and is polygenically inherited.

Animal production efficiency and quality increases by decreasing disease losses. Breeding for disease resistance can be performed alone or in combination with vaccination and medication. In breeding schemes, decisions have to be taken on what is supposed to be measured: general immune parameters, or disease incidences (natural or after on purpose / natural challenge). Disease resistance is difficult to measure on the selection candidates themselves. In a number of cases, measuring disease resistance involves challenges, which prevents further use in breeding. Selection for these traits needs to be based on measurements on relatives of the selection candidates.

Genetic selection against diseases must focus on increasing the bird's ability to response to disease challenge. Attention needs to be paid also to production efficiency as well. As indicated already by Gavora and Spencer (1983) selection for enhanced specific resistance to all diseases is impossible. On the other hand, an alternative for breeding against infectious diseases could be selection against disease susceptibility. Identification of susceptible animals might reduce the risk of infection disease outbreaks considerably. In this respect, the founder L line might represent a non-informative animal to identify gene regions necessary for immunocompetence as opposed to the H line.

Results in this thesis and the literature make it clear that disease resistance is heritable. Estimated heritability in chicken lines homozygous for MHC B19 selected for *Rous Sarcoma Virus* response (Pinard et al., 2004) was 0.46. An experiment dealing with resistance to ceacal carrier state of *Salmonella enteritidis* in chickens (Berthelot et al., 1998) showed an estimated heritability of 0.20. Antibody response to SRBC is also heritable (Martin et al., 1990; Siegel Gross 1980; Bovenhuis et al., 2002). The estimated heritability when using information of the selection lines was 0.17, and heritability estimated based on

the information from the F2 generation was 0.13. The estimated heritability, based on the current F2 generation, for other immune traits was low e.g. for KLH (0.07) but higher for LPS (0.17) and LTA (0.16). Although heritabilities are low, this still offers the possibility to select for increase overall immunity. The selected lines have proven that a very clear genetic change in this trait is possible within relatively small number of generations. As already presented in generation 10 of the founder lines of the current experiment populations, selection for High antibody response to SRBC increased overall immune responsiveness to different type of antigens and resulted in higher disease resistance to Marek Disease and Coccidiosis of the H line versus L line. Of a particular concern, however, might be negative correlation between antibody titers and production traits (Fulton, 2004). The selection of poultry for fast growth rate is most likely accompanied by a reduction in specific immune responses, with consequence increased disease susceptibility (Bayyari et al., 1997). Similar trade-offs between immunity and growth were reported in an experimental study in magpies (Soler et al., 2002), and in Japanese quail (Fair et al., 1999). For the founder lines of the current F_2 population a reciprocal situation was reported (Parmentier et al., 1996). The lines selected for immune response showed a trade-off in a BW gain what might be explained by a negative co-selection in the founder lines (Parmentier et al., 1996). Trade-off, however, might be avoided if multiple trait selection is applied. An antagonistic relation between two traits does not mean that simultaneous improvement is impossible but only that it is more difficult than for traits that show a favorable genetic correlation.

Information on QTL influencing disease resistance might be used to improve selection schemes. Information based on markers linked to QTL such schemes are referred to as Marker Assisted Selection (MAS). The benefits of MAS are expected to be largest for traits that are difficult to measure such as carcass quality and disease resistance. The anticipated additional gain of using genetic markers depends on the amount of genetic variation explained by QTL.

To obtain a more precise picture of the genetic basis of immune response and mutual regulation of different pathways, different experimental approaches could be used. At first less antigens could be applied, or these antigens should be grouped with regard to relation to immune pathways (Th1 or Th2). Secondly, related members of the same family might be used to define genetic regions related to immune response to various pathways. E.g. To be able to answer the question about genetic regulation of Th1 and Th2 responses, separate individuals of common origin should be immunized either with *M.butyricum,* or KLH.

In summary, the results in this thesis, indicate that differences in genetic background exist with regard to innate immunity. This gives opportunity for selection toward this trait. The innate immune response has thus far been neglected in breeding schemes. In general, it is proposed that the genetic basis of immune response should be analyzed at first at the level of innate immunity. Innate immunity gives an opportunity to observe the first line of defense.

Finally, this thesis gives very clear indications for the existence of QTL related with immune response to KLH on GGA14 although the founder lines had not been selected for this trait. This QTL was detected in the population described in this thesis, and subsequently validated in an independent population. This validation justified further efforts in fine mapping the QTL on GGA14 that resulted in narrowing down the region of interest to 0.1 cM. Given the strong immuno-modulating features of KLH, and the high level of Nabs towards KLH in (non immunized) poultry we propose to focus on the QTL on GGA14 by future studies.

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Summary

Given the growing concerns of consumers regarding the safety of animal-derived foods, it is important to develop effective methods to reduce chronic use of antibiotics and to enhance the effectiveness of vaccine protection by improving, via genetic selection, the innate ability of birds to respond to antigenic challenges. Immune responses to different pathogens follow distinct pathways and resistance to most diseases and pathogens is controlled by polygenes. The first genes studied as markers of immunity were the MHC genes; however, MHC genotypes explained only 3.5% of the total variation of antibody responses in chickens. Therefore the aim of the work described in this thesis is to identify genetic factors underlying immune response in laying hens. In addition, identification of genomic regions that underlie variation of immune (antibody) responses might add in the characterization of (unknown) immune mechanisms in poultry.

Selection lines used in the current study

The chickens, used in the presented experiments, originate from a cross (ISA Warren) between two divergently selected lines for either high (H line) or low (L line) primary (agglutinating) antibody responses to sheep red blood cells (SRBC). Selection in the founder lines was based on the individual total antibody titre at 5 days after primary intramuscular immunisation with SRBC at 37 days of age. In generation 10 and later generations, H and L line birds varied in various specific immune response characteristics. From the 17^{th} generation 15 males from the L line and 15 females from the H line; and 16 males from the H line and 16 females from the L line were selected to produce reciprocal crosses. Six randomly chosen F_1 males were mated to 12 randomly chosen F_1 dams to produce F_2 birds. Mating of males and females was such that inbreeding was avoided. Six hundred seventy two F_2 individuals were obtained, with an average of 56 offspring per full - sib family and 112 offspring per half – sib family.

Genetic basis of adaptive immune responses.

The chicken adaptive immune system involves humoral as well as cell-mediated responses. As is true for mammals, cell mediated immunity is regulated by CD3+ T cells probably via the release of different cytokines that results in either Th1 or Th2-like pathways.

The Th1 (inflammatory) response is stimulated by TNF, IFNγ and related cellular responses. The Th2 pathway is probably similar to mammals being stimulated by the cytokines IL4, IL5, and IL10 and results in a humoral response. Is has been proposed that chickens do not elicit typical Th1 responses, and that the chicken immune system lacks components which are under the regulation of the Th1 cytokines as in mammals. For this reason the chicken immune system may require less cytokines from the Th2 "class". In the current study a cellular type (Th1) response pathway was represented by *M. butyricum*, whereas humoral (Th2) response pathways were represented by SRBC, KLH and *E. coli*. In chapter 2 QTL detected for primary and secondary antibody responses to SRBC are described. The setup of the experiment allowed the use of two models to analyse the data: a paternal half-sib model where QTL alleles are traced to the F1 sire and a line-cross analysis model where QTL alleles are traced back to the founders of the selection lines. In addition the line cross analysis model gives the possibility to distinguish between mendelian QTL and parent of origin effects. In the half-sib analysis, two suggestive QTL for the SRBC primary antibody response were identified, first a QTL on GGA1, second a QTL on GGA16. In the line-cross analysis, seven QTL were identified. Three mendelian QTL were detected: on GGA4, GGA7, and GGA13. Four QTL with parent – of – origin effect were identified: on GGA2, GGA3, GGA27 and linkage group E60E04W23 for SRBC primary response a suggestive QTL was identified on GGA18 for SRBC secondary response. In chapter 3 a QTL analysis was performed on the data for two other antigens: KLH and *M.butyricum.* These analyses were performed on two independent populations: H/L cross and FP (feather pecking) lines. The second, FP population, is an F_2 cross between two commercial layer lines displaying differences in feather pecking behavior (FP). In the half-sib analysis, two QTL (on GGA14 and GGA3) were detected for the antibody response to KLH for the H/L population and three QTL (GGA12, GGA14 and GGA16) were detected for the FP population. Two QTL were detected for *M. butyricum* on GGA14 and GGA18 in the FP population using the half-sib analysis model. Three QTL were detected for the H/L population on GGA3, GGA13 and GGA14 using the line-cross analysis model. A QTL for the primary antibody response to KLH detected on GGA14 was validated in both populations under the half-sib analysis model. Based on the results differences in the genetic regulation of antibody responses to two different T-cell dependent antigens were suggested.

Genetic basis of innate immune responses.

In **chapter 4**, attention was focused towards the innate immune system. The question asked was; which genetic components of innate immune responses are identified and which relations between the innate and the adaptive immune system could be found.

Initially, the immune response towards any antigen is in the hands of the innate immune system. This is a subject of current intensive research in man and mouse after being overlooked for a couple of decades. Several complementary mechanisms are at play in innate immune responses, although the finesses (rules) are not fully understood at present. The "specific" parts of the innate immune system are formed by natural antibodies (NAb) which are present in non-immunised individuals. NAbs do not require induction of B-cells by on purpose antigenic challenge or mitogenic stimulation. NAb have low binding affinity and a broad specificity repertoire. In mammals, NAb are mainly of the IgM isotype, however, also IgA and IgG have been reported and are probably involved in early clearance of foreign material. The amount of NAbs increases with the age of the individual. The level of NAbs may be enhanced by either polyclonal stimulation, by exogenous microbes, by initiating responses of auto-reactive B cells or correspond with the secretion of naturally occurring auto – reactive B cell clones. The Natural antibodies in our studies were measured by level of antibodies binding the homotopes LPS and LTA in the two, before mentioned populations: H/L and FP cross. The QTL analysis was performed in two ways: a half - sib analysis, and a line – cross analysis. For the H/L population data were collected at two ages: 5 wks of age and 18 wks of age, whereas for the FP cross data were collected at 37 wks of age. In total 12 QTL were detected for non-specific antibody titers directed to LTA and LPS, and at 2 ages after in an F2 H/L population. For the antibody level binding LPS: QTL on GGA1, GGA4 (both ages), GGA3, GGA8, GGA12 and GGA18 were detected. For the antibody level binding LTA QTL on GGA2 (both ages), GGA3 (both models) and on GGA10 were detected. Similarly in an FP cross overall seven QTL were detected. For the antibody level directed to LTA QTL on GGA3, GGA7, two QTL for GGA14 (both models) were detected. For LPS QTL on GGA8, GGA18 and GGA27 were detected. These results indicate that different QTL regions are associated with levels of antibodies binding the homotopes LPS and LTA, respectively, in poultry.

QTL detection and beyond

In the whole genome scan approach microsatellite markers were used to identify QTL. However, when all available microsatellite markers were used, the identified region on GGA14 still spanned over 50cM (**chapter 3**). When working with model organisms or plants, strategies to improve the mapping resolution most often involve breeding a large number of progeny to increase the density of crossovers in the chromosomal region of interest. When working with humans or farm animals, this approach is not practical, and alternative strategies need to be applied. One approach that has recently received considerable attention is linkage disequilibrium (LD) mapping, which aims at exploiting historical recombinants. A promising approach in fine mapping a QTL region is combining information obtained by linkage analysis (LA) and linkage disequilibrium analysis (LD). In the LA approach information transmitted from the parents to their offspring is traced with DNA markers. If LA (roughly within family information) is combined with LD (roughly between family information), additional knowledge about historic recombination events is utilized. Closely linked markers provide more accurate information to the linkage analysis. In **chapter 5** an LDLA analysis was performed. Previously collected genotypic data was combined with additional information obtained by genotyping individuals with SNP (Single Nucleotide Polymorphism) markers. This analysis resulted in narrowing down QTL region from initial 50cM to the region below 1cM.

In the general discussion (**chapter 6**) the main findings from chapters 2 to 5 are used to discuss several issues: the biological nature of antigens used in this study and their mutual relations; genetic correlation between these antigens; QTL detected for both: innate and adaptive immune responses; fine mapping of one QTL region and possible candidate genes approach. Results in this thesis make it clear that disease resistance is heritable. The estimated heritability for SRBC primary responses when using information of the selection lines was 0.17, and heritability estimated based on the information from the F2 generation was 0.13. The estimated heritability, based on the current F2 generation, for other immune traits was low e.g. for KLH (0.07) but higher for LPS (0.17) and LTA (0.16). Although heritabilities are low, this still offers the possibility to select for increased overall humoral immunity. The selected lines have proven that a very clear genetic change in this trait is possible within relatively small number of generations. As already presented in generation 10 of the founder lines of the current experiment populations, selection for High antibody response to SRBC increased overall

immune responsiveness to different type of antigens and resulted in higher disease resistance to Marek Disease and coccidiosis of the H line versus L line. Detailed information on QTL influencing disease resistance might be used to improve selection schemes. The utilisation of information based on markers linked to QTL are referred to as Marker Assisted Selection (MAS). The benefits of MAS are expected to be largest for traits that are difficult to measure such as carcass quality and disease resistance. The anticipated additional gain of using genetic markers depends on the amount of genetic variation explained by QTL.

The results in this thesis indicate that differences in genetic background exist with regard to innate immunity. This gives opportunity for selection toward this trait. The innate immune response has thus far been neglected in breeding schemes. In summary, it is proposed that the genetic basis of immune response should be analyzed at first at the level of innate immunity, e.g. natural antibody levels (and possibly also complement components), whereas also the cellular part of innate immunity should not be neglected. Innate immunity gives an opportunity to observe the first line of defense, wheras it probably also determine subsequent specific immune responses for an important part.

Finally, this thesis gives very clear indications for the existence of QTL related with immune response to KLH on GGA14 although the founder lines had not been selected for this trait. This QTL was detected in the population described in this thesis, and subsequently validated in an independent population. This validation justified further efforts in fine mapping the QTL on GGA14 that resulted in narrowing down the region of interest below 1 cM. Given the strong immuno-modulating features of KLH, and the high level of Nabs towards KLH in (non immunized) poultry we propose to focus on the QTL on GGA14 for future studies.

Samenvatting

In de afgelopen jaren is de consument kritischer geworden als het gaat om voedsel van dierlijke afkomst. Binnen dit kader is het van belang om effectieve methoden te ontwikkelen om het gebruik van antibiotica te verminderen als mede een effectievere bescherming via vaccinaties van landbouwhuisdieren te stimuleren. Dit laatste kan bereikt worden door middel van genetische selectie op een effectievere respons van het aangeboren afweervermogen van een dier op een antigen.

De immuunrespons van een dier op verschillende pathogenen verloopt via verschillende routes. Resistentie tegen de meeste ziekten en pathogenen wordt geregeld via verschillende genen; zogenaamde polygenen. De eerste genen die als een genetische merker dienden, waren de major histocompatibility complex (MHC) genen. Echter, de MHC genotypen verklaren slechts 3,5% van de totale variatie van de antilichaamproductie in de kip. Het doel van het werk dat in dit proefschrift beschreven wordt, is het identificeren van de onderliggende genetische factoren voor immuniteit in legkippen. Identificatie van genomische regio's op het genoom van de kip kan meer inzicht geven in de bekende, dan wel onbekende mechanismen die aan de basis staan van de immuniteit van de kip.

Selectielijnen

De kippen die gebruikt zijn voor de experimenten zoals beschreven in dit proefschrift komen voort uit een kruising tussen twee geselecteerde kippenlijnen. Deze kippenlijnen zijn verschillend geselecteerd op basis van de primaire antilichaamrespons op schapen rode bloedcellen (SRBC). Een lijn is geselecteerd op een hoge respons (H-lijn) en de andere lijn is geselecteerd op een lage respons (L-lijn). De selectie in deze lijnen is gebaseerd op de antilichaamtiter op vijf dagen na i.m. immunizatie met SRBC op een leeftijd van 37 dagen. Na 10 generaties van selectie verschillen de H- en de L-lijn-dieren voor verschillende immuunrespons karakteristieken. In de 17^{e} generatie van de selectie zijn uit de H-lijn 16 hanen en 15 hennen en uit de L-lijn 15 hanen en 16 hennen random geselecteerd. Dit zijn de zogenaamde F0-dieren, welke dienen als basis voor een reciproce kruising. Dat wil zeggen dat een haan uit de H-lijn wordt gekruist met een hen uit de L-lijn en andersom. Uit de F1-generatie zijn er 6 hanen en 12 hennen random geselecteerd. Deze dieren vormen de basis voor de F2-generatie. De F2-generatie bestaat uit 672 dieren.

De genetische basis van de adaptieve immuunrespons

Het adaptieve (specifieke) immuunsysteem van de kip bestaat zowel uit een humorale als uit een cellulaire respons. In zoogdieren wordt de cellulaire immuniteit geregeld door CD3+ T-cellen via de secretie van verschillende cytokines. Deze cytokines zorgen ervoor dat de cellulaire respons via de Th1- danwel via de Th2-route verloopt. De Th1-respons wordt geregeld via cytokines als TNF, INFγ, en de gerelateerde cellulaire reacties. De Th2-respons bij de kip is waarschijnlijk vergelijkbaar met de Th2-respons bij zoogdieren en wordt geregeld via de cytokines IL4, IL5 en IL10. Deze Th2-respons resulteert in de zogenaamde humorale respons.
De kip vertoont geen typische Th1-respons zoals die bij zoogdieren wordt waargenomen. Het immuunsysteem van de kip lijkt geen componenten te bevatten die gereguleerd worden door Th1-cytokines in zoogdieren. In de huidige studie wordt *M. butyricum* gebruikt dat een cellulaire Th1-respons lijkt te induceren, terwijl de antigenen SRBC, keyhole limpet haemocyanin (KLH) en *E. coli* een Th2-respons oproepen.

In **hoofdstuk 2** worden de quantitatieve trait loci (QTL) voor de primaire en secundaire antilichaamrespons tegen SRBC beschreven. De opzet van deze studie maakt het mogelijk om de data te analyseren met behulp van twee verschillende statistische modellen, een ‘paternal half-sib model’ en een ‘line-cross model’. In het ‘paternal half-sib model’ worden de allelen vanuit de F2 terug getraceerd naar de F1-dieren, terwijl in het ‘line-cross model’ de allelen via de F1-dieren naar de grootouders (F0) worden getraceerd. Daarnaast biedt ‘line-cross model’ de mogelijkheid om onderscheid te maken tussen Mendeliaans overervende QTLs en QTLs onderhevig aan het ‘parent-of-origin’-effect. Het ‘parent-of-origin’-effect wil zeggen dat van een gen slechts het allel van één ouder tot expressie komt in het dier. De expressie van het allel verkregen van de andere ouder wordt onderdrukt. Met het ‘paternal half-sib model’ is een QTL gedetecteerd op kippenchromosoom (GGA)1 en ook op GGA16 voor de primairy antilichaamrespons tegen SRBC. Met behulp van het ‘line-cross model’ zijn er zeven QTLs gedetecteerd voor de primaire antilichaam respons tegen SRBC. Drie Mendeliaans overervende QTLs op respectievelijk GGA4, GGA7 en GGA13 en vier QTLs met een ‘parent-of origin’-effect op respectievelijk GGA2, GGA3,GGA27 en E60E04W23. Daarnaast is er een QTL voor de secundaire antilichaamrespons tegen SRBC op GGA18 gevonden.

In **hoofdstuk 3** wordt de QTL analyse beschreven voor twee andere antigenen: KLH en *M. butyricum*. Deze studie is in twee ongerelateerde populaties uitgevoerd, in de HL-kruising en de FP-kruising. De FP-populatie bestaat uit een kruising van twee commerciële Witte Leghorn-lijnen. Met behulp van het 'paternal half-sib model' zijn er voor de antilichaamrespons tegen KLH twee QTLs (GGA14 en GGA3) in de HL-populatie gedetecteerd en drie QTLs (GGA12, GGA14 en GGA16) in de FP-populatie gedetecteerd. Voor *M. butyricum* zijn er twee QTLs (GGA14 en GGA18) gedetecteerd in de FP-populatie met behulp van het 'paternal half-sib model'. In de HL-populatie zijn drie QTLs (GGA3, GGA13 en GGA14) gedetecteerd met behulp van het 'line-cross model'. Het QTL voor KLH op GGA14 is daarmee gevalideerd in twee onafhankelijke kippenpopulaties.

De genetische basis van de aangeboren immuunrespons

In **hoofdstuk 4** wordt er gekeken naar het aangeboren immuunsysteem met als vraagstelling: kunnen er QTLs gedetecteerd worden voor de aangeboren immuniteit en wat is de relatie met tussen aangeboren en adaptieve immuniteit.

Elke respons, op wat voor antigen dan ook, wordt in eerste instantie geregeld door de aangeboren immuniteit. Decenia lang werd er weinig aandacht besteed aan de rol van de aangeboren immuniteit bij de antilichaamrespons. Echter, nu is het weer een belangrijk onderzoeksonderwerp in de huidige immunologie van mens en muis. Verschillende mechanismen spelen een rol in de regulatie van de aangeboren immuniteit, maar de exacte regels waarmee het proces verloopt is nog onduidelijk. De antilichamen van de aangeboren immuniteit worden gevormd door de zogenaamde 'natuurlijke antilichamen' (Nabs) die voorkomen in niet-geïmmuniseerde individuen. Nabs hebben geen inductie nodig van B-cellen door bijvoorbeeld immunisatie met een specifiek antigen of via mitogene stimulatie. Nabs hebben een lage bindingsaffiniteit met een breed skala aan antigenen.

In zoogdieren zijn Nabs voornamelijk van het IgM-isotype, maar er zijn ook IgA- en IgG-isotypes gevonden. Het niveau van de Nabs stijgt met de leeftijd van een dier. Dit wordt waarschijnlijk veroorzaakt door polyclonale stimulatie via exogene microben of door de initiatie respons van auto-reactieve B-cellen. De Nabs in deze studie worden gemeten door de hoeveelheid antilichamen die gebonden worden aan de homotopen lipopolysaccharides (LPS) en lipoteichoic acid (LTA). In de HL-populatie is de LPS- en de LTA-titer gemeten toen de dieren 5 weken en 18 weken oud waren. In de FP-populatie ware de dieren 37

weken oud. De QTL-analyse is uitgevoerd zoals reeds beschreven in deze samenvatting. Voor de binding van de Nab aan LPS zijn er verschillende QTLs gedetecteerd in de HL-populatie. Te weten een QTL op GGA1, GGA3, GGA8, GGA12 en op GGA18. Voor de binding van Nabs aan LTA zijn er QTLs gedetecteerd op GGA2, GGA3 en GGA10. Voor de binding van de Nabs aan LPS in de FP-populatie zijn er QTLs gedetecteerd op GGA8, GGA18 en GGA27. Voor de binding van Nab aan LTA zijn er QTLs gedetecteerd op GGA3, GGA7 en GGA14. De resultaten duiden erop dat er verschillende QTL-regio's op het kippengenoom een rol spelen voor de Nab bindingscapaciteit aan LPS en LTA.

Het detecteren van QTLs en daarna

In de methode die tot dusver gebruikt is voor de QTL-detectie zijn microsateliet-merkers gebruikt. Het nadeel van deze methode is dat de dichtheid van deze merkers op het genoom niet dicht genoeg is om een hele precieze locatie aan te wijzen waar het QTL (= gen) zich bevindt. Bijvoorbeeld de regio op GGA14 (zie hoofdstuk 3) spreidt zich uit over een lengte van 50 cM op het chromosoom. In modelorganismen zoals muis en plant kunnen grote hoeveelheden nakomelingen verkregen worden uit een kruising. Hiermee wordt de mapping resolutie verbeterd doordat het aan tal cross-overs in het genoom toeneemt. Maar wanneer er met mens of landbouwhuisdieren gewerkt wordt, behoort dit niet altijd tot de mogelijkheden. Een nieuwe methode die recentelijk meer aandacht krijgt, is de zogenaamde linkage disequilibrium (LD)-mapping. Deze methode neemt de informatie uit voorgaande generaties mee in de analyse. Wanneer LD gecombineerd wordt met linkage analyse (LA) is het mogelijk om een betere schatting te maken van de positie van het QTL op het chromosoom. In **hoofdstuk 5** wordt de LDLA-analyse beschreven en toegepast op het QTL voor KLH op GGA14. De QTL-regio wordt teruggebracht van 50 cM naar minder dan 1 cM.

In de algemene discussie (**Hoofdstuk 6**) worden de resultaten van hoofdsuk 2 tot en met 5 bediscussieerd, waarbij de volgende aspecten aan de orde komen: de biologische aard van de antigenen die gebruikt zijn, de genetische correlatie tussen de respons op deze antigenen, de QTLs die gevonden zijn voor zowel de adaptieve als de aangeboren immuunrespons en het 'fine-mappen' van het QTL op GGA14 en de mogelijke kandidaatgen-benadering. De resultaten beschreven in dit proefschrift laten zien dat ziekteresistentie erfelijk is. De erfelijkheidsgraad (h^2) gebaseerd op de selectielijnen (H-lijn en L-lijn) is 0.17 terwijl de h^2

gebaseerd op de HL-populatie 0.13 is. De h^2 gebaseerd op de HL-populatie voor KLH is 0.07, voor LPS 0.17 en voor LTA 0.16. Hoewel deze h^2 –en laag zijn, bieden deze enige ruimte om te selecteren op algemene humorale immuniteit. De selectielijnen hebben laten zien dat het mogelijk is om in een klein aantal generaties een groot verschil te verkijgen tussen de de twee selectielijnen in de immuunrespons tegen SRBC. Selectie op een hoge antilichaamtiter tegen SRBC geeft ook een toename in de algemene immuniteit tegen Marek's Disease en coccidiosis ten opzichte van de L-lijn-dieren.

Informatie over QTLs voor ziekteresistentie kunnen huidige selectieschema's verbeteren. Deze informatie kan gebruikt worden door de specifieke merkerinformatie te gebruiken in een fokprogramma. Dit is de zogenaamde merker ondersteunende selectie (MAS). Het gebruik van MAS komt het best tot zijn recht bij kenmerken die moeilijk te meten zijn, een lage erfelijkheidsgraad hebben of maar in één sexe tot expressie komen. Daarnaast hangt het voordeel van gebruik van QTL informatie sterk af van het effect van het QTL.

De resultaten beschreven in dit proefschrift laten zien dat verschillen in genetische achtergrond bestaan voor aangeboren immuniteit. Dit biedt de mogelijkheid tot selectie op dit kenmerk. Aangeboren immuniteit (bijv. NAbs) wordt tot dusver niet gebruikt in fokprogramma's. Een voorstel gebaseerd op resultaten uit dit proefschrift is, dat de genetische basis van de immuniteit eerst geanalyseerd moet worden op het niveau van de aangeboren immuniteit. Daarbij moet gekeken worden naar Nab-niveau's (als mede ook naar complementniveaus'/activatie), en het cellulaire aspect van de aangeboren immuniteit.

Dit proefschrift geeft duidelijke aanwijzingen voor een QTL voor de respons tegen KLH op GGA14. Dit QTL werd gevalideerd in twee onafhankelijke kippenpopulaties. Fine-mappen van dit QTL resulteerde in een verkleining van de QTL regio tot minder dan 1 cM. Gegeven de sterke immuno-modulatie-eigenschappen van KLH en de hoge Nab response tegen KLH in niet geïmmuniseerde kippen, verdient het QTL op GGA14 extra aandacht in toekomstige studies.

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I, Maria Siwek, was born on 20 of may 1969 in Poznan (Poland). In 1983 I completed my education on Agriculture University in Poznan, specialization of Biotechnology and Animal Breeding and obtained Msc degree. After the graduation I got a job position as a research assistant in Institute of Zootechnic in Zakrzewo near Poznan. During several years which I spent in this Institute I was involved in projects related with genetic analysis of local polish breeds of laying hens and gees. The other interest of mine, was towards the possibility to create chicken chimeras using blastodermal germ cells and primordial germ cells. In 1996 I stayed for a 6 months training period in Wageningen University, Department of Animal Breeding and Genetics. This stay resulted in mine further cooperation with the Animal Breeding and Genetics Group and realization of the phd project.

Nawoord

And this is it. After four years and five months spent in zodiac, here I ‘m. I did enjoy my project, I did enjoy work at ABG department and I did enjoy getting to know dutch language (a bit) dutch culture and habits. It is time to move one, with new experience and knowledge.

The first people to thank are my supervisors: Jan van der Poel and Henk Parmentier for constant support, faith and lots of fun we had during our discussions.
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M.

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Completed PhD Education Plan			**Graduate School WIAS**	
Name PhD student	Maria Siwek			
Group	ABG			
Daily supervisor(s)	Jan van der Poel + Henk Parmentier			
Supervisor(s)	Johan van Arendonk			
Project term	2000	2004		
The Basic Package (minimum 2 cp)			year	cp
WIAS Common Course (2 cp)			2000	2.0
Course on philosophy of science and/or ethics (mandatory)			2001	1.0
Subtotal Basic Package				3.0
Scientific Exposure (conferences, seminars and presentations, minimum 5 cp)			year	cp
International conferences (minimum 2 cp)				
WCGALP			2002	1.0
EAAP			2002	1.0
EAAP			2003	1.0
NE-1016			2003	1.0
NE-1016			2004	1.0
Candidate Gene			2002	1.0
EPGS			2003	1.0
Seminars and workshops				
MAS - QTL			2004	1.0
ROLDUC			2002	1.0
ROLDUC			2003	1.0
ROLDUC			2004	1.0
Presentations (minimum 4 original presentations of which at least 1 oral, 0.5 cp each)				
WCGALP - oral			2002	0.5
EAAP - oral presentation			2003	0.5
EAAP - oral presentation			2004	0.5
NE-1016 oral presentation			2003	0.5
NE-1016 oral presentation			2004	0.5
MAS - QTL oral presentation			2004	0.5
EPGS poster presentation			2003	0.5
ISAG - poster presentation			2002	0.5
Subtotal International Exposure				15.0
In-Depth Studies (minimum 4 cp)			year	cp
iRNA			2004	0.5
Undergraduate courses (only in case of deficiencies)				
QTL analysis				1.0
Subtotal In-Depth Studies				1.5
Professional Skills Support Courses (minimum 2 cp)			year	cp
WIAS Course Techniques for Scientific Writing (advised)			2002	2.0
Use of Laboratory Animals (mandatory when working with animals, 3 cp)			2001	3.0
Subtotal Professional Skills Support Courses				5.0
Didactic Skills Training (optional)			year	cp
Genomics - assisting in Msc course			2003	1.0
Genomics - assisting in Msc course			2004	1.0
Robert Deerenberg - supervising practical			2002	1.0
Yonash Fessehaye- supervising practical			2003	1.0
Magdalena Guzowska - supervising Msc thesis				1.0
Claudia Cangemi - supervising Msc thesis				1.0
Subtotal Didactic Skills Training				6.0
Education and Training Total (minimum 21 cp, maximum 42 cp)				30.5

* one credit point (cp) equals a study load of approximately 40 hours

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