Gene expression profiling of chicken intestinal host responses

Promotoren:

Prof. dr. M.A.M. Groenen

Persoonlijk hoogleraar bij de leerstoelgroep Fokkerij en Genetica, Wageningen Universiteit

Prof. dr. M.A. Smits

Persoonlijk hoogleraar bij de Animal Sciences Group, Wageningen UR

Copromotor:

Dr. J.M.J. Rebel

Projectleider, Animal Sciences Group, Wageningen UR

Promotiecommissie:

Prof. dr. M.R. Muller	(Wageningen Universiteit)
Prof. dr. T.A. Niewold	(Katholieke Universiteit Leuven, België)
Prof. dr. ir. H.F.J. Savelkoul	(Wageningen Universiteit)
Prof. dr. J.A. Wagenaar	(Universiteit Utrecht)

Saskia van Hemert

Gene expression profiling of chicken intestinal host responses

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op woensdag 31 januari 2007
des namiddags te half twee in de Aula.

Gene expression profiling of chicken intestinal host responses

Gen expressie profielen van gastheer reacties in de kippendarm

Ph.D. Thesis – Saskia van Hemert, 2007
Animal Sciences Group of Wageningen UR, Division infectious diseases

With summaries in English and Dutch

ISBN 90-8504-581-9

Abstract

Chicken lines differ in genetic disease susceptibility. The scope of the research described in this thesis was to identify genes involved in genetic disease resistance in the chicken intestine. Therefore gene expression in the jejunum was investigated using a microarray approach. An intestine specific cDNA microarray was generated from a normalized and subtracted library. Gene expression in young chickens was studied using two different disease models, malabsorption syndrome and *Salmonella enteritidis*. For each model two different chicken lines were studied, which differed in susceptibility to the specific diseases. Gene expression differences between the chicken lines were found under control and under infected conditions. In the studies described here the main focus was on genes that could be involved in disease susceptibility. Large differences between the chicken lines with different genetic backgrounds were found in their gene expression responses to the infections. After malabsorption syndrome the more susceptible chicken line regulated immune related genes, genes involved in food absorption and genes with unknown functions. The chicken line most susceptible for salmonella upregulated genes involved in inflammation, or with unknown functions, whereas the more resistant chicken line regulated genes involved in acute phase response, the fibrinogen system, actin polymerisation, and also genes with unknown functions. Most gene expression responses to both infection models were found 1 day post infection. Gene expression differences between the two chicken lines lead to the hypothesis that immunological differences could be the basis of differences in susceptibility for *Salmonella*. Therefore the two chicken lines were studied for the phagocytic properties of intestinal mononuclear cells and these properties were different for the two chicken lines. Also, a decrease in the number of $CD4^+$ T-cells and macrophages in response to the *Salmonella* infection was found only in one chicken line. In both chicken lines the number of $CD8^+$ T-cells increased, but faster in the susceptible chicken line. So genetic background influences intestinal gene expression responses and immunological responses.

Contents

Chapter 1.

General introduction

Introduction

Chickens are the most abundant birds in the world with a population of more than 24 billion. They are mainly kept for egg and meat production. Genetic selection of chicken has been primarily based on these production traits. This has led to two different breeds of chickens: egg laying chickens (layers) and meat-type chickens (broilers). Nearly all commercial raised broilers originate from one of the four main breeding companies in the world. Yearly 43 million broilers are produced in the Netherlands of which about 500,000 are organic (free range) broilers. Organic broilers are not slaughtered before 81 days of age whereas regular broilers reach their slaughter weight nowadays in approximately 6 weeks.

Important economic losses for the poultry industry can occur, due to for instance intestinal infectious diseases (101). For example salmonellosis in young chickens results in losses of approximately US$64 million to US$114 million annually in the United States (26). To prevent (intestinal) infections in poultry, antibiotics were widespread used. However, the preventive use of antibiotics is now forbidden in the countries of the European Union. Therefore other ways to improve (intestinal) health should be developed, for example by improving genetic disease resistance. In this research project a genomics approach has been used to identify chicken genes encoding for proteins involved in determining genetic susceptibility to intestinal infectious diseases.

The gastro-intestinal tract

The overall function of the gastrointestinal tract (GI-tract) is to process ingested feed into molecular forms that can be transferred from the external environment to the body's internal environment, along with salts and water. In addition the GI-tract has an important function to protect the body against pathogens, thus there is an overall balance between feed absorption and protection against foreign antigens. The wall of the GI-tract has the general structure illustrated in Figure 1.1.

Extending from the surface of the small intestine are villi which in turn are covered by microvilli to increase the surface area available for absorption. The surface is covered by a single layer of epithelial cells and the epithelial cell layer is covered on the lumen side with mucus produced by goblet cells. The epithelial cells are linked together along the edges of their luminal surfaces by tight junctions. Epithelial cells are continuously replaced by new epithelial cells. New cells arise by mitosis from cells at the base of the villi in the crypts. The new cells differentiate as they migrate to the top of a villus. Just below the epithelium is a layer of connective tissue, the lamina propria.

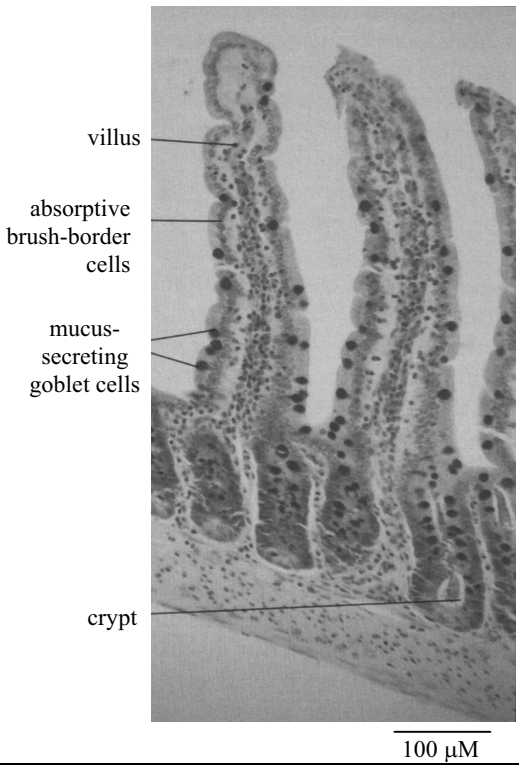

Figure 1.1. Photograph of a section of part of the lining of the small intestine (4).

Besides the functions in digestion and absorption, the GI-tract is also a major barrier between external and internal environments. Therefore crucial immunological defence systems are active in the intestine. All intestinal cells involved in immunological defences in the GI-tract are together known as gut-associated lymphoid tissues (GALT). The GALT is one of the major immunological systems of the body (160). The GALT comprises all cells and tissues along the alimentary tract and in chickens include organized lymphoid structures, such as the bursa of Fabricius, cecal tonsils, Peyer's patches, Meckel's diverticulum and lymphocyte aggregates scattered along the intra-epithelium and lamina propria of the GI-tract (101). The bursa of Fabricius is the central lymphoid organ for B-cell lymphocyte maturation, where antibody diversity is generated. In the cecal tonsils both T- and B-cells are present. The precise role of the cecal tonsils is unknown, but a role in antigen sampling has been suggested (17). The Peyer's patches are lymphoid aggregates in the intestine which possess a morphologically distinct organized lymphoepithelium, containing B-cells and

T-cells (17). Peyer's patches in chickens have many of the characteristics of mammalian Peyer's patches. In chicken the abundance and distribution of Peyer's patches vary with age. They are not evident at hatching, but are identified 10 days post-hatching. In adult chickens only one Peyer's patch is consistently found (17). Meckel's diverticulum is a remnant of the yolk on the small intestine and it contains germinal centers with B-cells and macrophages.

The GI-tract is colonized by a commensal microflora. The composition of the commensal microflora can be influenced by probiotics, dietary supplements containing potentially beneficial bacteria like lactic acid bacteria. Also prebiotics can influence the composition of the commensal microflora. Prebiotics are most often carbohydrates like oligosaccharides, which are non-digestible food ingredients that improve host health by selectively stimulating the growth or activity of a limited number of beneficial bacteria in the intestine. The gastrointestinal epithelium can tolerate a significant degree of bacterial colonization.

Chicken intestinal diseases

The GI-tract is a portal of entry for many pathogens. Infections of the GI-tract commonly occur in chickens of all age groups, but tend to predominate in young birds as the GI-tract is not yet fully developed in young chickens (64). There are many pathogens that replicate in the intestine of poultry, like *Escherichia coli*, *Salmonella enteritidis*, *Campylobacter jejuni*, *Eimeria spp.* or rotavirus. Malabsorption syndrome is also an important intestinal disorder in chickens, but the exact infectious agents are not known.

Different enteric bacterial pathogens may have different effects on the intestinal epithelium: alterations in the structure and function of the tight junction barrier, induction of fluid and electrolyte secretion, and activation or inhibition of the inflammatory cascade (18). To prevent intestinal diseases in poultry, it is important to understand the host responses to intestinal pathogens and to understand the avian intestinal immune system, but the exact host response after interaction with a pathogen are not all known yet. Infections of the GI-tract are known to negatively impact poultry production and negatively influence the well-being of the chickens. Also, infections of the gastrointestinal tract likely contribute to the development of other diseases. For instance pathogen induced mucosal damage may provide a portal of entry for other potential pathogens. Also diseases due to nutritional deficiencies can occur as a result of a gastrointestinal infection when feed absorption is affected (64).

Different ways for disease prevention and control exist: vaccine protection against known antigens, preventive use of antibiotics, pharmacological intervention against

pathogens, and hygiene measurements. For the development of prevention and control strategies, most attention has been given to the pathogen so far and only limited attention has been given to the resistance of the host itself. Since only a few effective vaccines for the control of enteric diseases are available, the poultry industry has adopted widespread use of antibiotics to control disease and to maintain bird health. However, in the European Union the widespread use of antibiotics (as growth promoters) has recently been forbidden (January 2006). The main reason was that the long-term use of antibiotics has caused public concern regarding the effect on environmental sustainability and development of antibiotic resistance in human bacterial pathogens. On the other hand there is an increased demand for safe food products, free from zoonotic pathogens such as *Salmonella spp.* and *Campylobacter spp*. Another way for disease prevention, besides the use of antibiotics, is modulation of intestinal defence by feed ingredients or the use of pro- or prebiotics. Selecting animals for disease resistance may also affect disease incidence in a positive manner. For food safety issues and disease control it will be beneficial to increase the genetic disease resistance in animals. So far breeding for disease resistance has not been applied in chickens, but it has been applied on a limited scale in other species, like resistance to scapie in sheep and to enterotoxigenic *E. coli* in pigs (15, 181).

Malabsorption syndrome

Malabsorption syndrome (MAS) affects the chicken intestine and is one of the subjects of this thesis. MAS is an acute enteric disease affecting fast growing broilers and is also called infectious stunting syndrome or runting and stunting syndrome. The disease is characterised by the widespread occurrence of stunting and uneven growth in a flock with a high culling rate, wet litter, retarded feathering, diarrhoea with undigested feed, pigment loss and bone abnormalities. MAS affected chickens develop severe enteritis with cystic deformation of the crypts of Lieberkühn and atrophy of the villus (53, 142). MAS develops during the first three weeks of age (115, 143) and the jejunum is the part of the intestine that is affected most by MAS.

The aetiology is associated to infection of the gut by a group of enterogenic viruses and bacteria. Despite many efforts to elucidate the exact cause(s) of MAS, the aetiology of MAS was not established. From intestinal homogenates of MAS--affected chickens reovirus, haemolytic *E. coli*, *Pasteurella hemolytica* and *Enterococcus durans* could be isolated. None of these pathogens alone reproduced MAS in broilers (171, 173). It was suggested that reovirus in combination with substance(s) in the intestinal homogenates play a role in weight gain depression (173). Haemolytic *E. coli* in combination with reovirus and formalin treated homogenate from MAS affected intestines did not induce weight gain depression although this combination caused intestinal lesions, but less severe than the MAS homogenate itself. Thus far, a single

causal agent for MAS could not be established and therefore MAS is recognised as a multifactorial syndrome.

Susceptibility to MAS differs between broiler lines based on differences in severity of the intestinal lesions and the reduction in body weight gain (reviewed in (140)). This indicates that there are genetic factors influencing susceptibility to MAS, but so far these factors are largely unknown. The susceptibility is correlated with apoptosis and heterophil infiltration of the jejunum (210). Heterophils are the avian equivalent of mammalian neutrophils. Susceptibility to MAS is also associated with the number of $CD4^+$ and $CD8^+$ T-cells in the intestinal villi and the mRNA expression levels of various cytokines under both control and MAS induced conditions (139). No relation was found between susceptibility to MAS and the development of the intestine, liver, bursa of Fabricius or spleen (209).

Salmonella

Salmonella enterica is an enteropathogenic bacterial species that in humans can cause a variety of syndromes ranging from common food poisoning to the sometimes life-threatening typhoid fever. The type of disease caused by these bacteria not only depends on the serovar of the infecting bacteria but also on the host species and its immunological status. Intestinal cells are the first host cells to interact with ingested enteric pathogens. They are key for the invasion of salmonella into the mucosa and, subsequently, spread to other organs (55). In chicken *Salmonella enterica* serovars Typhimurium and Enteritidis are capable of causing severe systemic disease in newly hatched chicks and in birds under extreme stress conditions. The infection seldom causes mortality in birds more than 1 month old (176). In young chickens infection with salmonella leads to clinical signs of systemic disease, diarrhoea and dehydration, intestinal lesions and to an influx of heterophils into the gut accompanied by inflammation and damage to villi (13). Heterophils play a key role in protecting chickens from the development of systemic disease following infection with *Salmonella* serovar Enteritidis by largely restricting the bacteria to the gut (89).

The outcome of an encounter between salmonella and its host is dependent upon multiple factors including the host genetic background. To study the genetic factors involved in resistance to this pathogen, mouse models for salmonella infection have widely been studied (113). Several genes and pathways have been identified that may influence the disease outcome, like genes of the MHC complex, *NRAMP1* and Toll-like receptor 4 (reviewed in (95, 151)). Chickens have also been used to study salmonella susceptibility, because in addition to its impact on human health, salmonellosis in young chickens results in economic losses for the poultry industry

(26). Research on the identification of quantitative trait loci (QTL) and other association studies (see below, genetic variation) in chickens have revealed considerable differences between chicken lines in levels of colonization of salmonella of the GI-tract and in responses to vaccination (97, 106). In this respect, it is important to clearly define resistance to salmonella in chickens, as an inverse relationship between severity of caecal infection and colonization in systemic organs has been suggested (91, 154). A general mechanism of resistance might apply to all serotypes of *Salmonella enterica* in chickens, as lines that had previously been shown to be resistant to *S. enterica* serovar Typhimurium were also found to be resistant to the serovars Gallinarum, Pullorum, and Enteritidis (28). Resistance to salmonellosis in chicken is a polygenic phenomenon, with multiple genes involved as demonstrated in a number of studies (reviewed in (195)).

The immune system

The immune system is the host defence system against infectious diseases. Any immune response involves recognition of the pathogen or other foreign material and mounting a reaction against it to eliminate it. The immune responses can be divided in two linked systems, innate immunity and adaptive immunity. The early phase of a response, without prior exposure to a pathogen, depends on the innate immunity, whereas the adaptive immunity depends on specific responses of antigen specific lymphocytes, which are developed upon and after exposure to an antigen. The innate immune system is capable of removing the infectious agents shortly after the infection through direct killing of the pathogen. The innate immune system also activates and regulates immune reactions of both the innate and the adaptive immune system by antigen presentation and production of effector molecules like cytokines. Elements of the innate immune system include: constant peristaltic flushing, barrier of mucus, the presence of bile salts and organic acids, antimicrobial peptides and defensins, resident microflora, cytokines, macrophages, heterophils, NK-cells and the complement system. One of the mechanisms by which the innate immune system senses the invasion of pathogenic micro-organisms is through receptors that recognize specific molecular patterns that are present in microbial components, for instance toll-like receptors. The family of Toll-like receptors recognise patters, like LPS, flagellins, peptido-glycan, dsRNA and CpG DNA (3). This makes the innate immune response not as a-specific as was thought previously. Stimulation of toll-like receptors not only leads to the activation of innate immunity, but also instructs the development of antigen-specific adaptive immunity.

The adaptive immune system is antigen specific and has two important effector mechanisms to attack pathogens. One is based on the formation of immunoglobulins by

B-cells and is called humoral immune response. The other, the cellular response, is executed by T-cells. A variety of T-cells exists, each with their own individual effector mechanism. So are $CD8^+$ T-cells cytotoxic T-cells that kill infected target cells and $CD4^+$ T-cells are helper T-cells that mainly activate macrophages and B-cells. Genetic selection for improved broiler performance has resulted in a decrease in the humoral immune response, but an increase in the cell-mediated and inflammatory responses (34).

In the intestine, multiple cell types are present that are involved in immune responses, like intestinal epithelial cells (IECs), lymphocytes and macrophages. IECs have traditionally been regarded as passive cells primarily responsible for maintaining the integrity of the intestinal barrier. However, it is now widely appreciated that they are also important regulators of innate and acquired immunity (37, 134). IECs secrete chemicals harmful to bacteria, viruses, and parasites and provide early signals important for initiation and regulation of the inflammatory response following invasions at the intestinal surface. IECs produce several important cytokines including IL-1, IL-6, IL-8 TNF-α, and granulocyte macrophage-colony stimulating factor. Other immunomodulatory factors produced by IECs are complement components, antimicrobial peptides, defensins and prostaglandins (37).

Lymphocytes are located on two anatomic compartments in the intestine, in the epithelium and the lamina propria. Lymphocytes located in the epithelium are mainly T-cells, which are generally located close to the villus core (79). The lymphocyte population in the lamina propria is relatively enriched with immunoglobulin-producing B-cells (101).

Intestinal macrophages regulate inflammatory responses to bacteria and antigens that have reached the epithelium, protect the mucosa against harmful pathogens, and scavenge dead cells and foreign debris. As effector cells macrophages are part of the innate immune system. In the intestine macrophages are mostly located in the lamina propria. In the non-inflamed intestinal mucosa macrophages are non-inflammatory, but they retain host defence functions (169). These non-inflammatory macrophages are an example that the intestine does not induce an immune response to all passing antigens like micro-organisms and dietary products, a feature that is known as oral tolerance.

Following exposure to pathogenic microbes, significant architectural and other changes in the intestine occur, which include increased permeability, infiltration of cells, increased proliferation of crypt cells, and increased production of mucin, enzymes, and immoglobulines (Igs). Complex interactions between lymphocytes, epithelial cells, dendritic cells, and resident macrophages are involved in both secretory

Ig and mucin production during the host defence to generate a microenvironment incompatible with pathogen survival.

Differences in disease susceptibility

Natural disease resistance refers to the inherent capacity of an animal to resist disease when exposed to pathogens, without prior exposure or immunization. Different read-out parameters can be used to measure disease resistance, like mortality, survival time, fever, loss of production, transmission, and the amount of pathogens able to colonize the body. In any population, differences in disease resistance or susceptibility between individuals are common. In chicken, differences in disease susceptibility are found for a number of diseases, like Marek's disease, avian leucosis, infectious bronchitis, infectious bursal disease, salmonellosis and coccidiosis (27). Disease susceptibility, like many other economically important traits in farm animals, is attributed by genetic and environmental components.

Genetic variation

Disease susceptibility is for a large part heritable and therefore genetically determined. Some breeds or strains are inherently resistant or less affected by a pathogen that can be fatal to other members of the same species. Genetic disease resistance can be investigated by mapping disease resistance loci by linkage analysis in inbred lines. Quantitative trait loci (QTLs) are stretches of DNA that are closely linked to the genes that underlie the trait in question. QTLs can be located in the genome through associations between phenotypes and the inheritance of genetic markers in a suitable pedigree (32). To date, more than 600 QTLs have been described in chicken for a variety of traits, such as growth, body weight, carcass composition, egg production, fatness, ascites, feather pecking, stress and disease resistance (31, 42). The recently developed single-nucleotide polymorphism (SNP) map is an important tool to fine map QTLs (200). Chickens have a high degree of genetic diversity with SNP rates between and within chicken lines of about 5 SNPs per 1000 base pairs. This is six- to sevenfold larger than in humans and domestic dogs, and threefold larger than in gorillas (200). The SNP sequence data indicated that there are similar degrees of difference between different breeds of modern chickens and between those chickens and the red jungle fowl (considered as the nearest ancestor to the domestic chicken). Around 70% of the SNPs were common to all breeds, suggesting that most of the nucleotide diversity must have originated before the domestication of chickens, 5000-10,000 years ago.

In chicken, QTL studies have been conducted for resistance to Marek's disease virus, salmonellosis and coccidiosis. In addition, QTLs affecting antibody response to Newcastle disease virus and *E. coli* have been mapped (93). Such linkage studies are

very labour intensive, as extensive breeding is required to establish inbred lines with contrasting phenotypes. For complex traits, with multiple genes involved, many different QTLs can be found. Further, the resolution that is usually obtained is limited, so the QTL region still contains many genes. In addition, different QTLs for the same trait may be identified when different starting populations are used, due to different genetic variations in the investigated populations.

Genetic disease resistance can also be investigated by association studies in populations that have been propagated by random mating for at least 20 generations (93). These studies may identify markers associated with traits, and these markers are either tightly linked to a trait or are responsible for the trait itself. A disadvantage is that significant associations might be found by chance, due to the multiplicity of tests. In most association studies the analysis is restricted to candidate genes that may potentially be involved in disease resistance. For example, twelve candidate genes involved in the pathogenesis of salmonella infection in chicken were recently tested for their association with salmonella load in caecum, spleen or liver (90). Eleven of the twelve selected genes had a significant association with one of the investigated phenotypic traits. Choices for candidate genes are biased, because the choice is based on the known biological properties of the gene products, phenotypes associated with mutant alleles in other biological systems, or by their location in a QTL region as determined by linkage analysis.

For disease resistance most candidate genes that have been postulated are involved in immune mechanisms. Genetic differences in disease susceptibility can be due to the diversity of the immune system and variation in immune reactivity during infection. An immune response can be ineffective due to a too low, too high, or a misdirected reaction. Genetic variation in immune responses has been linked to the structural and functional diversity of the MHC complex, immunoglobulins, cytokines and other proteins (for chicken, reviewed in (211)).

Besides the variation of the immune system also other genetic variations can affect disease susceptibility. An example is the F4 receptor in the intestine of pigs. Particular enterotoxigenic *E. coli* can only adhere when the F4 receptor is expressed (181). Pigs without F4 receptor are therefore not susceptible to these enterotoxigenic *E. coli*. Also in chickens receptors are involved in disease susceptibility, like receptors for Mareks disease virus (10).

To investigate genetic disease susceptibility at a molecular level, traditionally one gene at a time was studied, mostly by a candidate gene approach. *NRAMP1* (presently known as *SLC11A1*) was the first gene identified to be involved in disease resistance in mice (reviewed in (23, 61)). *SLC11A1* is expressed in macrophages and is associated with resistance to mycobacterium, leishmania and salmonella infections. Also in chicken *SLC11A1* has been linked to disease resistance (72). The MHC gene family

has long been the subject of intense investigation for its role in disease resistance in chicken. Other candidate genes in chickens with a proven or potential role in disease resistance are: cytokine genes, CD-encoding genes, T cell receptor genes, growth hormone, transforming growth factors, caspase 1, inducible nitric oxide production and the immunoglobulin genes (90, 96).

Environmental conditions

Stress, management conditions, nutrition, and age are examples of environmental conditions that may influence disease susceptibility. Stress is known to affect immune functions and infectious disease susceptibility in both humans and animals (109). The central stress response generally inhibits innate immune responses, whereas the peripheral nervous system tends to amplify local innate immune responses (175). In chicken different kinds of stress are described that influence the immune system: social stress (aggressive pecks and fights), cold and heat stress, feed restriction, the lightning schedule and stocking density (41, 65, 66). Feed restricted birds show a reduction of *in vitro* lymphocyte proliferation and lymphoid organ weights, compared with birds fed ad libitum, suggesting higher disease susceptibility (66).

Also hatching conditions can influence disease susceptibility in chickens. Factors influencing the hatching process include storage conditions of the eggs, incubation time, temperature and relative humidity of the incubator and the brooding temperature. For example, different studies have indicated that one day of storage of the egg before hatching negatively influences viability compared to fresh eggs or 2 days of storage (50, 136, 145).

Nutrition may have an effect on the development of the immune response. Dietary components as fatty acids, vitamins or minerals, and amino acid composition influence the immune function in chickens (86). The diet may also impact the incidence of infections by its functional characteristics in the lumen of the GI-tract. Physical and chemical aspects of the diet can modify the populations of micro-organisms in the GI-tract, the capacity of pathogens to attach to enterocytes and the integrity of the intestinal epithelium (87).

Also the condition of the mother hen can influence disease susceptibility of the offspring. Disease susceptibility in the chicks can be affected by altering nutrient levels in the diet of mother hens or with *in ovo* nutrient administration (86, 141). For instance the number of leukocytes increased in broilers descended from hens receiving additional vitamins and trace minerals (141). Furthermore the age of the mother hen can influence disease susceptibility of the offspring. In general chicks from younger hens are less fit than those from older hens (165).

Also the age of the infected individual influences disease susceptibility. In general, young individuals are more susceptible to infectious diseases compared to older ones,

due to an immature immune system. In neonatal chickens the secondary immune organs, like spleen, caecal tonsils and Peyer's patches, are not entirely developed as these organs have an incomplete structural organisation (111). The chick's GI-tract undergoes dramatic changes within the first few days of life. In the days following hatching small intestinal weight increases more rapidly in relation to body weight than other organs (132). A rapid increase in mass, villi number and length, enterocyte number, crypt depth and proliferating cells occurs in these first days. Also the lymphocyte populations in the gut develop and differentiate during this period. (100). Shortly after hatch the gut is poorly populated by both innate immune leukocytes and lymphocytes (54). Also the microflora at hatch is quite different from the colonized gut. High levels of inflammatory responses were observed following an infection of newly hatched chicks whereas these responses were not observed following infection of birds of 1 week old (199).

Gene expression and disease susceptibility

Pathogens are able to modulate and interfere with the transcriptional program of host cells and have developed a variety of strategies and molecular machinery to accomplish this (5, 77). The genes targeted by pathogens frequently belong to the innate immune system, or are involved in host cellular processes. Processes of the host that can be modulated by pathogens are for example cell cycle progression, actin cytoskeleton rearrangements or modulation of secretory pathways.

Recognition of a pathogen causes changes in the gene expression levels in particular cells of the host, the primary host response. These changes in gene expression levels can be different between susceptible and resistant chickens. Several examples have been described in the literature. For instance gene expression differences were found between the lymphocytes of chickens susceptible or resistant to Marek's disease after the induction of the disease (104). Chickens from a MAS-resistant line had higher mRNA concentrations for IL-2, IL-6, IL-18 and IFN-gamma in the small intestine than chickens from a MAS-susceptible line (139). After induction of MAS the relative amounts of intestinal IL-2, IL-6, IL-8 and IFN-gamma mRNA increased more in the susceptible line than in the resistant line. After exposure to salmonella, pro-inflammatory cytokine mRNAs (IL-6, IL-8, and IL-18) were found to be up regulated in heterophils from salmonella-resistant chicks compared to susceptible chicks. Furthermore, heterophils from the resistant chickens had significantly decreased mRNA expression levels of transforming growth factor-β4, an anti-inflammatory cytokine, when compared to heterophils from susceptible chickens (52, 178). In a study to the caecal carrier state in chickens for salmonella, a lower expression of the IFN-γ gene was observed in the susceptible infected animals compared to the resistant ones and healthy counterparts (154). In addition a high

baseline level of defensin gene expression was recorded in young animals from the susceptible line irrespective of the infection (154). This demonstrates that gene expression differences between susceptible and resistant chicken lines might already be detectable under control conditions.

A disadvantage of the approaches indicated above is that only one or a limited number of genes are investigated and not their mutual relationships. Resistance to most diseases is a complex trait which is almost certainly controlled by multiple genes. A single QTL or gene cannot account for the observed differences in susceptibility. Therefore investigation of the expression levels of multiple genes would be interesting. mRNA profiling with the aid of microarrays can be used to study gene expression levels of multiple genes in a single experiment.

During the study described in this thesis, the draft sequence of the whole chicken genome became available (74). The chicken represents the first agricultural animal to have its genome sequenced and the chicken genome bridges the evolutionary gap between mammals and other vertebrates. The whole genome of the chicken is composed of approximately one billion base pairs of sequence and an estimated 20,000 - 23,000 genes. The chicken genome size is very compact, two to three times smaller than the human and mouse genome, although the number of estimated genes is only slightly lower than for human and mouse. When the chicken genome is compared to other sequenced genomes, prudence is called for concluding that avian homologs to mammalian genes do not exist, as several genes known from expressed sequence tags (ESTs) were not identified in the first chicken genome assembly. It is estimated that up to 10% of the chicken genes are still missing from the current assembly. A major problem for gene annotation in chicken using mammalian sequences, in particular for genes involved in immunity, is the lack of sequence homology between these genes due to their high rate of evolution (31, 80). However, sequencing of the chicken genome has also identified new immune related genes, like interleukins, type I interferons, chemokines and tumour necrosis factor superfamily members (80). The sequence information of the whole chicken genome has recently been used to generate whole genome microarrays.

Microarrays are a collection of microscopic DNA spots attached to a solid surface, such as glass, plastic or silicon chips. The affixed DNA segments are known as probes. On microarrays either oligonucleotides or cDNA probes are printed, representing thousands of genes. To obtain genome-scale expression data, mRNA from the source of interest is converted to fluorescently labelled cDNA and hybridised to the microarray. Most often gene expression profiles from two different situations, e.g. infected versus non-infected, are compared. The two mRNA samples are labelled with a different fluorphore, one with a green (cy-3) and the other with a red (cy-5) label, and

hybridised simultaneously on the microarray. A green spot indicates higher expression levels of that gene in the first sample while a red spot indicates higher expression levels in the second sample. Yellow spots indicate equal expression levels in both samples. Instead of hybridising two samples and compare gene expression differences also only a single sample can be hybridised on a microarray. In that case the intensities of the spot can be compared with the intensities obtained with another microarray hybridised with another sample. The resulting image obtained with a fluorescence scanner or phorphorimager is processed with computer software to generate a spreadsheet of relative or absolute gene expression values. Sophisticated algorithms and statistical methods are applied to normalize the hybridisation data in such a way that the expression ratios or levels for each DNA element can be determined. The collective data provide a gene expression profile associated with the physiological, pathological or genetic condition under study.

Differences in levels of gene expression following a disease might provide insight into mechanisms of disease susceptibility. For example, in sheep over one hundred genes were differentially expressed in the duodenum between lambs genetically resistant and susceptible for gastrointestinal nematodes as measured with a 10k bovine cDNA microarray (44). Pathway analysis revealed that genes differentially expressed in resistant animals were those involved in the development of an acquired immune response and those related to the structure of the intestine smooth muscle. In another study, using a 20k ovine cDNA microarray, 41 genes were identified to be differentially expressed between susceptible and resistant animals in the absence of infection. However the observed expression differences were low, less then twofold. The identified genes could be related to a stress response as well as to a variety of other functions (85), indicating that changes in gene expression can be broader than only affecting immune genes. Another example of this is the up regulation of kinases and transcription factors in the human epithelial cell line HT-29 after exposure to salmonella (48). Recently it was revealed that ubiquicidin, some ribosomal proteins, some histones and phospholipase A_2 have antibacterial activity in addition to their basic cell function (47). Therefore broad gene expression studies might reveal additional proteins with defence properties besides the classical immune genes.

Objective and outline of the thesis

Chicken lines vary in their susceptibility to infectious diseases. However, genes and proteins involved in disease resistance are not known yet. The scope of this thesis is to identify genes involved in genetic disease resistance and to get insight into the mechanisms that determine differences in susceptibility. Therefore gene expression profiles were measured in intestinal tissues derived from chickens that differ in their susceptibility to intestinal infectious diseases. These studies might lead to the

identification of genes involved in disease resistance, a better understanding of the underlying mechanisms, and to the development of read-out parameters for intestinal health. The latter might be used not only in breeding programs but also in attempts to optimize intestinal health by modulation of environmental factors or nutrition.

To study genes involved in disease resistance a normalized and subtracted chicken intestine specific cDNA library and microarray was developed (chapter 2). In a hybridisation experiment the microarray was tested for its ability to identify up- and down regulated genes in MAS infected chickens. Gene expression after a MAS induction was further investigated in chapter 3 where gene expression was investigated in two different chicken lines that differed in susceptibility to MAS. To investigate whether there are common genes involved in disease susceptibility a new experiment was performed with another disease model, salmonellosis. Gene expression differences between two chicken lines differing in salmonella susceptibility were investigated (chapter 4). In chapter 5 the list of gene expression differences between the two chicken lines was extended by the use of a whole genome microarray instead of the intestine specific cDNA microarray. The results of the whole genome microarray were compared with the results of the home made microarray described in chapter 2. In addition genes are described that are induced by salmonella, irrespective of the susceptibility of the chicken line. In chapter 6 gene expression differences between chicken lines in response to a salmonella infection were correlated with immunological differences. Therefore the phagocytic properties of intestinal mononuclear cells were investigated. Also the number of intestinal $CD4^+$ T-cells, $CD8^+$ T-cells and macrophages was examined by immunohistochemical methods. In addition, RNA expression levels in the jejunum were studied. In the general discussion (chapter 7) the findings and observations are summarised and discussed in a broader context.

Chapter 2.

Generation of EST and microarray resources for functional genomic studies on chicken intestinal health

Saskia van Hemert
Bastiaan Ebbelaar
Mari Smits
Annemarie Rebel

Animal Biotechnology (2003) 14: 133-143

Abstract

Expressed sequenced tags (ESTs) and microarray resources have a great impact on the ability to study host response in mice and humans. Unfortunately, these resources are not yet available for domestic farm animals. The aim of this study was to provide genomic resources to study chicken intestinal health, in particular malabsorption syndrome (MAS), which affects mainly the intestine. Therefore a normalized and subtracted cDNA library containing more than 7000 clones was prepared. Randomly chosen clones were sequenced for control purposes. New ESTs were found and multiple ESTs not identified in the chicken intestine before were observed. The number of non-specific ESTs in this cDNA library was low. Based on this normalised and subtracted library a cDNA microarray was made. In a preliminary hybridisation experiment with the microarray, genes were identified to be up- or downregulated in MAS infected chicken. These genes are likely to be related to infection of the intestine and therefore to MAS. This indicates that the generated resources are valuable tools to investigate chicken intestinal health by whole genome expression analysis approaches.

Introduction

Malabsorption syndrome (MAS) is a worldwide distributed disease and affects broilers in the first few weeks of age. It is a multifactorial disease and the exact agents causing the disease are not yet known, but different viruses and bacteria are characterised (173). The disease is characterised by weight gain depression with non-uniform growth, defective feathering, and diarrhoea with undigested food and watery content. Most lesions are present in the digestive organs, in particular in the small intestine. Experimental reproduction of MAS can be done by oral inoculation of 1-day-old broilers with homogenates obtained from digestive tissues of affected chicken. Because MAS mainly affects the intestine, it can be used as a model to study intestinal health and intestinal disturbances in young broilers.

Identifying potential important genes involved in chicken intestinal health may be done with a number of techniques like QTL, differential display, SNP detection and microarrays (104, 122, 124, 129, 167, 206). cDNA microarrays are a recommended technique to study mRNA expression profiles of many different genes simultaneously (118). A functional genomics approach would be useful to identify genes or cellular processes involved in a MAS infection and perhaps also to other enteric disorders in chickens. However, the resources for a functional genomics approach for chicken intestinal health are not yet available. Microarrays that contain large collections of expressed sequence tags (ESTs) from chickens and other domestic animals are, in contrast to human, rat and mouse not (commercially) available, but the devices to build a microarray from a cDNA library are available.

In this study, the construction and analysis of a normalized and subtracted chicken jejunum specific cDNA library is described. Based on this library a cDNA microarray was generated and it is demonstrated that this microarray can be used to reproducibly detect gene expression differences between infected and control chicken intestines.

Material and Methods

Chicken

One-day old chicks of 2 different broiler breeder lines were used in the present study (B and D in (209), obtained from Nutreco®, Boxmeer, The Netherlands). Eighteen chicks of each line were randomly divided into 2 groups, 9 chicks each. At day 0, one group was orally inoculated with 0.5 ml of the MAS-homogenate (homogenate C in (172)) and the other was the control group, orally inoculated with 0.5 ml Dulbecco's phosphate buffered saline (PBS). Three chicks of each group were randomly chosen and sacrificed at day 1, 4 and 13 post inoculation (pi) and tissue

samples were collected. Pieces of the jejunum and breast muscle were snap frozen in liquid nitrogen and kept at -70°C until further use.

RNA Isolation

Pieces of the jejunum and breast muscle tissue were crushed under liquid nitrogen. 50-100 mg tissues of the different chicks were used to isolate total RNA using TRIzol reagent (GibcoBRL), according to instructions of the manufacturer with an additional step. The homogenised tissue samples were solved in 1 ml of TRIzol Reagent using a syringe and needle 21G passing the lysate for 10 times. After homogenisation, insoluble material was removed from the homogenate by centrifugation at 12,000 × g for 10 minutes at 4°C.

Two pools of total RNA were made, one from jejunum RNA and one with muscle RNA, using equal amounts of RNA from the different chicks and different time points. The two total RNA pools were used to isolate poly(A)$^+$ RNA using Qiagen Oligotex Minikit (Qiagen) following the manufacturer's protocol.

Construction of the cDNA Library and Expressed Sequence Tags

A custom-subtracted cDNA library was made using the PCR-select cDNA subtraction kit (CLONTECH) according to the manufacturer's protocol. Jejunum cDNA was used as tester and breast muscle was used as driver in the subtraction. A PCR analysis of the subtraction efficiency was performed using glyceraldehyde phosphate dehydrogenase (GAPDH) primers (5'-AGCAGCAGCCTTCACTACC-3' and 5'-TGGGCACGCCATCACTATCTTCC-3') to amplify a fragment in subtracted as well as unsubtracted jejunum cDNA. One reaction of 60 µl contained 150 ng cDNA, 2.4 µl of both GAPDH-primers(10 pmol/µl), 6 µl of 10× ExTaq buffer (TaKaRa), 1.2 µl dNTP mixture (2.5 mM each, TaKaRa) and 0.05 µl TaKaRa ExTaq (5 units/µl). The PCR conditions were 33× {94°C for 30 sec, 60°C for 30 sec, 68°C for 2 minutes}. After 19, 23, 28 and 33 cycles 5 µl from each reaction was removed and analysed on a 1% agarose gel.

After subtraction, the cDNA pool was incubated for 20 minutes at 70°C using Taq polymerase to generate an A-tail. cDNA was ligated into the pGEM-T easy vector, using the pGEM-T Easy kit (Promega) following instructions and ligation mix was transformed to XL-2 blue ultracompetent cells (Stratagene) and the bacteria were plated out.

PCR, Sequencing and Sequence Analysis

83 randomly chosen clones were picked and a PCR reaction was performed. One reaction of 20 µl contained: 2 µl of 10× ExTaq buffer (TaKaRa), 0.4 µl dNTP mixture (2.5 mM each, TaKaRa), 0.06 µl nested primer 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and nested primer 2 (5'-AGCGTGGTCGCGGCCGAGGT-3', 100 pmol/µl), 0.05 µl TaKaRa ExTaq (5 units/µl), 17.43 µl sterilised distilled water and a bacteria clone from the library. The PCR was performed using a thermocycler (Primus) programmed to conduct the following cycles: 2 min 95°C, 40× {10 sec 95°C, 30 sec 69°C, 90 sec 72°C}, 5 min 72°C. The PCR amplification products were purified using Sephadex G50 fine column filtration.

3 µl of the purified PCR product was sequenced using 10 pmol of nested primer 1 and 4 µl of ABI PRISM BigDye Terminator Cycle Sequencing Ready reaction in a total volume of 10 µl. The sequence reaction consisted of 2 min 96°C, 40× {10 sec 96°C, 4 min 60°C}. Sequencing was performed on an ABI 3700 DNA sequencer. Sequence results were analysed using SeqMan 5.00 (Dnastar). The sequences were compared with a chickenEST database (http://www.chick.umist.ac.uk/cgi-bin/chicken_database.cgi) consisting of 350,000 ESTs from 21 different chicken tissues (25). Sequence similarity of cDNA was also tested against the GenBank non-redundant nucleotide library (http://www.ncbi.nlm.nih.gov/BLAST/), using the Blastn option and when no significant hits were found the Blastx option (6).

Clones whose sequences exhibited similarities to database sequences, with E-values lower than 1E-20 were operationally classified as known chicken genes. Sequences with E values lower than 1E-5 with the Blastx option were considered as homologous to known genes of other organisms.

Construction of the Microarray

First a screening PCR reaction was performed with the conditions described above. The PCR amplification products were analysed on a 1% agarose gel. Clones with a vector without an insert and clones that gave multiple products with the PCR reaction were removed from the library. The remaining clones were used to receive the cDNA to spot on a microarray by performing a PCR in 200 µl reaction mixture containing a small amount of bacteria, increasing all PCR reagents with a factor 10. Temperature conditions consisted of an initial denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 45 sec, annealing at 69°C for 45 sec and

elongation at 72°C for 2 min, and then a final elongation at 72°C for 10 minutes. The PCR products were again analysed on a 1% agarose gel and were purified using a Sephadex G-50 fine column filtration. An isopropanol precipitation was performed and the products were dissolved in 15 µl spotting buffer (50% DMSO, 0.15 M PO_4-buffer pH 8.5).

A cDNA microarray was made by spotting 3072 ESTs in duplo on a CMT-GAPS^{TM} coated slide (Corning) with a spot diameter of 150 µm and a distance between spots of 300 µm. The slides were heated for 4 hours at 80°C before use.

Hybridising of the Microarray

Before hybridisation, the microarray was pre-hybridised in 5% SSC, 0.1% SDS and 1% BSA at 42°C for 30 minutes. To hybridise the microarray the MICROMAX TSA labelling and detection kit (PerkinElmer) was used. The TSA probe labelling and array hybridisation were performed as described in the instruction manual with minor modifications. Biotin- and fluorescein-labelled cDNAs were generated from 5 µg of total RNA from chicken jejunum per reaction. The cDNA synthesis time was increased to 3 hours at 42°C, as suggested (84). Post-hybridisation washes were performed according to the manufacturer's recommendations. Hybridisations were repeated with the fluorphores reversed. After signal amplification the microarrays were dried and scanned in a GeneTAC2000 (Genomic Solutions). The image was processed (geneTAC software, Genomic Solutions) and spots located and integrated with the spotting file of the robot. Reports were created of total spot information and spot intensity ratio for subsequent data analyses.

Analysis of the Microarray Data

After background correction the data were presented in an M/A plot were $M=\log_2 R/G$ and $A=\log_2 \sqrt{(R \times G)}$(46). An intensity-dependent normalisation was performed using the lowess function in the statistical software package R (203). The normalisation was done with a fraction of 0.2 on all data points.

Results

Chicken Jejunum cDNA Library

The subtraction efficiency during the cDNA library construction was checked using a PCR analysis. A GAPDH PCR product is visible after 28 cycles in the subtracted jejunum sample, while it is already visible after 19 cycles in the

unsubtracted jejunum sample (Figure 1), indicating that mRNA concentration of this housekeeping gene is decreased in the subtracted sample.

A total of 78 × 96 independent clones were amplified. The inserts of the first 34 × 96 clones showed a range in size from 100 to 1400 bp (Figure 2). About 10% of the analysed clones did not contain detectable inserts or gave multiple bands and they were removed from the library. 83 randomly chosen clones were sequenced to check diversity of the library. These 83 sequences could be divided into 71 different contigs (Table 1). The 71 contig-sequences were compared with the sequences present in the NCBI database by Blast analysis. 18 sequences (25%) were identified as known chicken genes, 25 sequences (35%) showed significant homology with known genes from other organisms and the remaining 28 sequences (40%) showed no significant similarity with known genes. One sequence without similarity with known genes was found 3 times and two sequences were found twice. Some different sequences happened to be from the same gene. From 16S rRNA three different parts were found in the library and from aldolase B, anterior gradient 2 and structural polyprotein avian nephritis virus two different parts were found.

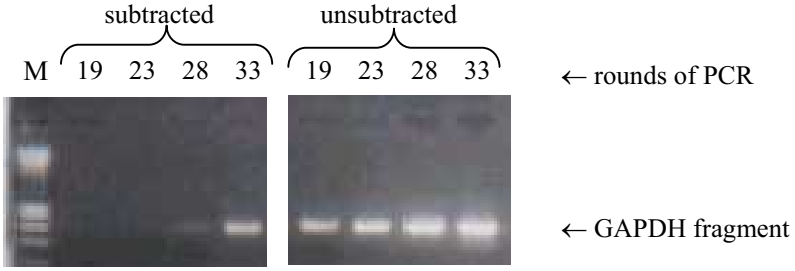

Figure 2.1: Agarose gel electrophoresis of GAPDH fragment after different rounds of PCR amplification, as indicated. Unsubtracted jejunum cDNA and subtracted jejunum cDNA are used as input in the PCR. *Key:* M = marker.

Table 1. Genes present among 83 random clones from the library using the NCBI blast search..

Times found	Name of the gene/ homologue	Times found	Name of the gene/ homologue
6	*16S rRNA* [1]		
2	*Lysozyme G*	2	Liver-expressed antimicrobial peptide 2
2	Interferon-inducible peptide		
1	*16S rRNA* [2]	1	Epithelial chloride channel protein
1	*16S rRNA* [3]	1	Gamma1-adaptin
1	*a-actine*	1	Ganglioside-induced differentiation ass. prot.3
1	*Aldolase B* [4]	1	Guanylin
1	*Aldolase B* [5]	1	Hes 1
1	*b-actine*	1	Hypothetical protein
1	*Cytochrome P450*	1	Hypothetical protein human
1	*Galectin-2*	1	Interferon-induced membrane protein
1	*IL15*	1	KIAA0342 gene product
1	*Interferon regulatory factor 2*	1	Legumain
1	*Keratin-19*	1	Microsomal triglyceride transfer protein
1	*NADH cytochrome b5 reductase*	1	Ribosomal 12S
1	*Ovomucin alpha-subunit*	1	RIKEN cDNA 1200003J11 gene
1	*Na/K-transporting ATP-ase*	1	Secreted protein HT protein
1	*Tubulin*	1	Sim. to gene near HD on 4p16.3 S.pombe
1	*Vit.D induced calbindin-D28 gene*	1	Similar to hypothetical protein FLJ1
1	Anterior gradient 2 [6]	1	Structural polyprotein avian nephritis virus [8]
1	Anterior gradient 2 [7]	1	Structural polyprotein avian nephitis virus [9]
1	Calcium-sensitive chloride channel	1	Unknown protein
1	Coatomer protein complex, subunit β1		

Italic = chicken gene

1 = nucleotide 3612-3854 mitochondrial genome
2 = nucleotide 3466-3580 mitochondrial genome
3 = nucleotide 3851-3915 mitochondrial genome
4 = nucleotide 6480-6663 aldolase B
5 = nucleotide 7152-7264 aldolase B
6 = aminoacid 34-101 anterior gradient 2
7 = aminoacid 127-167 anterior gradient 2
8 = nucleotide 5904-6168 avian nephritis virus gene for structural polyprotein
9 = nucleotide 6526-6705 avian nephritis virus gene for structural polyprotein

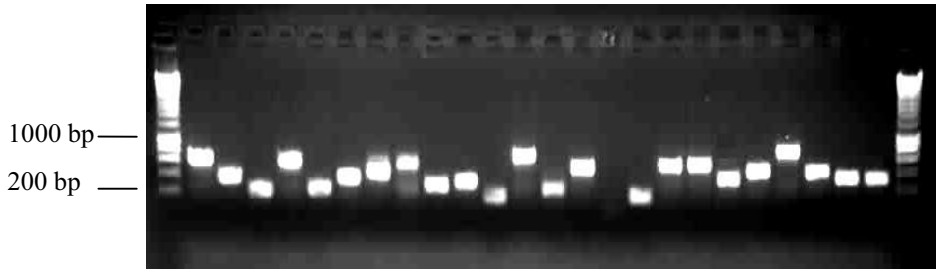

Figure 2.2: Agarose gel electrophoresis of PCR-amplified cDNA from chicken intestine after extracting the empty and double bands. The numbers to the left indicate the position of the DNA size standards (Smartladder, Eurogentec).

Tissue Distribution of Sequenced ESTs

The 71 different sequences were compared with the chickenEST database of UMIST/Nottingham/Dundee universities consisting of 350,000 chicken ESTs using Blast analysis. Based on this comparison and on the tissue distribution of the ESTs that gave significant hits five different groups could be formed:

ESTs found only in the small intestine

ESTs found in the small intestine and other tissue(s)

ESTs found in the small intestine, muscle and other tissues

ESTs found in other tissues than small intestine

ESTs with no hits in the database.

The distribution of the ESTs over the different groups is shown in Table 2.

Table 2. Tissue distribution of ESTs in the library, based on 83 clones.

Tissues with a BLAST hit	Times found	Percentage of total
Only small intestine	9	13%
Small intestine + other tissue(s)	23	32%
Small intestine + muscle + other tissue(s)	8	11%
Other tissue(s), no small intestine	20	28%
No hits	11	16%

The amount of sequences aligning with ESTs found in many different tissues is low, only 11%. A significant percentage of the library contains intestinal specific inserts (45%).

From the 11 sequences that gave no hit with the chickenEST database, two encode for the structural polyprotein of the avian nephritis virus. The remaining 9 sequences, which gave no hit with the chickenEST database were compared with the NCBI EST database. One sequence gave a hit with a clone from a chicken liver library and another with a clone from a chicken Bursa of Fabricius library. The rest of the sequences gave no hit and these 7 sequences are considered as new EST sequences, so about 10% of the library contains unknown gene sequences.

mRNA Expression Profiling

In a preliminary experiment, a comparison was made between MAS-infected and uninfected chickens. A pool of jejunum RNA from five MAS-infected chickens from one broiler line at day 5 post infection was compared to a pool of RNA from five non-infected chickens from the same line at day 5. As expected, more than 84% of the 6144 spots on the microarray showed significant hybridisation, with both Cy3 and Cy5 signals more than 2 times above the background. The hybridisation was repeated with the Cy3 and Cy5 labels reversed. The cDNA was spotted in duplo on each slide. A gene was considered up- or downregulated when there were at least 3 measurements and all measurements gave a ratio higher than 3. In the MAS infected chicken 37 cDNAs (1.2%), were upregulated more than 4-fold and 82 cDNAs (2.7%) more than 3-fold as shown by a change in the Cy3/Cy5 ratio. 37 cDNAs (1,2%) were downregulated more than 4-fold and 59 (1.9%) more than 3-fold. Some of the clones corresponding to these spots were sequenced and six sequences with a high homology to known genes were found (Table 3). The whole experiment was repeated to investigate the reproducibility. The data from the up and down regulated genes from the two experiments were comparable, the ratios found in the two experiments differed by a median of 0.68.

Table 3. Up- and downregulated genes in MAS infected chicken at day 5 post infection.

log_2 (inf/contr)	description	GenBank accession No.	Blastn/ blastx	E-value	Organism
+5.1	Lysozyme G	P00718	blastx	4e-31	*Ostrich*
+3.1	Interferon induced membrane protein	NP_776976	blastx	6e-14	*Bos Taurus*
+2.1	Interferon induced 6-16 protein	A26316	blastx	9e-6	*Homo sapiens*
-2.6	Cytochrome P450	CAB62060	blastn	0	*Gallus gallus*
-3.1	Calbindin	P04354	blastn	0	*Gallus gallus*
-3.3	Apolipoprotein B	P11682	blastn	e-149	*Gallus gallus*

Note: An e-value of 0 means that there is no change that the hit is found by coincidence.

Discussion

The development of high-quality integrated resources, such as microarrays, is required for functional genomics approaches to investigate complex problems in animal and veterinary science (204). In this study, such resources were developed with a focus on the chicken jejunum in order to allow studies on chicken intestinal health. In addition, it was demonstrated that these resources can succesfully be used to identify differences in mRNA expression profiles between control and challenged animals.

For the cDNA library construction a subtraction and normalisation was performed to obtain intestinal specific genes and to increase rare transcripts. The subtraction was sufficient for our purposes, although the subtracted sample still contained some cDNAs that correspond to mRNAs common to both the intestine and the breast muscle, which was used for subtraction. This was shown by the GAPDH PCR in subtracted and non subtracted samples.

Almost half of the sequenced clones in the library contained intestine-specific ESTs and the amount of ESTs expressed in a large amount of different tissues is low. The intestine-specific ESTs gave hits with ESTs from the database found in the small intestine only, or in the small intestine and some other tissues, often liver, kidney + adrenal gland, and/or whole chick embryos. Only 11% of the library gave hits with ESTs from the database found in many different tissues, including muscle that was used for the subtraction. This means that the performed subtraction was not 100%, although only a small part of the library contained ESTs expressed in many different tissues.

In general the library showed good diversity as was determined by sequencing 83 randomly chosen clones. However, eight of the sequenced clones coded for three different parts of 16S ribosomal RNA. The same was found by Carré *et al.* (33). This is the result of reverse transcription initiated from an A-rich region inside the rRNA sequence rather than from the poly(A) tail at the 3'end of the RNA, used to create the library. Although some ribosomal RNA could be expected, the percentage found is quite high. However, this library will be used to create a cDNA microarray and the large amount of spots on this array will overcome the high percentage of 16S ribosomal RNA.

As expected, the library contains known chicken genes as well as so far unidentified chicken genes. The chicken genome is not yet completely sequenced. The percentage of sequences without similarity to known genes (40%) is comparable with earlier results (33).

Interestingly, 10% of the ESTs in this library were unidentified and 28% of the ESTs were not described in the intestine before. The unidentified ESTs are usually considered as novel genes. However, our ESTs were generated from a random part of the cDNA whereas most other chicken EST sequences that are present in the databases are derived from the 5' end (33). Therefore, it is possible that these ESTs correspond to the 3' part of genes already identified, but without deposition of 3' end sequences in databases. This possibility is confirmed by finding 2 different parts of the anterior gradient 2 and structural polyprotein avian nephitis virus genes. On the other hand, infected broilers and healthy broilers were used in this study for cDNA construction whereas for the chicken EST database, all tissues were taken from healthy White Leghorn (Layer) breed only. So it might be that the novel ESTs correspond to novel chicken genes, which are upregulated during an infection or to genes specific for broilers.

To see whether or not these resources can be used to study chicken intestinal health, gene expression profiles of MAS infected chickens and uninfected chickens were compared. Different broiler lines differ in their susceptibility for MAS as is measured by a difference in growth retardation and the amount of lesions in the intestine after experimentally induced MAS (171, 209). Therefore a MAS susceptible line is used to investigate MAS. The hybridisation was repeated with the dyes reversed,

to filter out artifacts that could be attributed to unequal incorporation or quenching of the dye molecules (2) and all cDNAs were spotted in duplo on a slide. Almost 150 of the 3072 cDNAs showed a more than 3-fold up- or downregulation.

Some of the up- or downregulated genes could easily be related to intestinal infection and problems with absorption. Upregulated genes are lysozyme G and two interferon induced proteins. Lysozyme G is expressed in heterophils (128) and heterophils are shown to infiltrate tissue after an infection (67). Interferon is involved in different immune responses. Thus these upregulated genes are known to be involved in infections and can therefore be involved in the MAS-infection. Downregulated genes are apolipoprotein B, calbindin and cytochrome P450. Apoliprotein B is involved in lipid transport, calbindin is a vitamine D-dependent calcium binding protein and cytochrome P450 is involved in metabolism. So all three proteins are involved in the digestion and absorption of food. Therefore these genes can be involved in MAS, because this syndrome affects the absorption properties of the intestine. The rest of the spots are under investigation.

Differentially expressed genes can be detected using this described cDNA microarray as shown by the result of this experiment. Therefore these resources can now be used to study chicken intestinal health, for example susceptibility for intestine-related diseases, intestinal development, intestinal immunology and the progress of a disease in time.

Chapter 3.

Differences in intestinal gene expression profiles in broiler lines varying in susceptibility to malabsorption syndrome

Saskia van Hemert
Arjan Hoekman
Mari Smits
Annemarie Rebel

Poultry Science (2004) 83: 1675-1682

Abstract

Examination of the host gene expression response upon encounter with pathogens may provide insight into the cellular events following an infection. In addition it may shed light on the basic mechanisms underlying differences in the susceptibility of the host. In this study gene expression in the chicken jejunum was investigated in two different broiler lines under control and malabsorption syndrome (MAS) affected conditions. The two broiler lines differ in their susceptibility for MAS. The gene expression was investigated at six different timepoints post inoculation using a custom made intestine specific cDNA microarray. More than 70 up- or downregulated genes were identified after a MAS inoculation in both broiler lines. However, the number of the up- and downregulated genes varied between the two lines, with more differences in expression in the most susceptible line. Marked differences were observed in expression profiles between the two broiler lines, in control as well as in the MAS affected animals. The microarray data were validated and confirmed by quantitative RT-PCR. The differentially expressed genes included immune related genes, genes associated with food absorption and genes that need to be characterized further before their role in MAS and MAS susceptibility can be understood.

Introduction

Broiler strains can differ in susceptibility to infectious diseases which is probably due to their genetic differences (96, 211). During selection of strains, disease susceptibility was given minor attention, whereas major attention was given to specific economical traits as feed conversion and growth rate. Breeding chickens with an improved resistance against different infectious diseases would be beneficial, because the use of antibiotics is under pressure and will be forbidden in the near future. Therefore it would be useful to consider genes involved in disease susceptibility as a trait in new breeding programs (57).

Genes associated with disease susceptibility may be discovered by comparing on a genome-wide scale susceptible and less susceptible lines under control and challenge conditions (104, 206). Identifying potential important genes for disease susceptibility in chickens may be done with a number of different techniques like QTL (Quantitative Trait Loci), differential display, SNP detection, microarrays or a combination of these techniques (104, 122, 124, 129, 167, 206). cDNA microarrays are a recommended technique to study mRNA expression profiles of many different genes simultaneously (118). Nowadays expressed sequence tags (ESTs) from chickens are available in the public database and microarrays have been generated, including a chicken jejunum cDNA microarray (183).

Malabsorption syndrome (MAS) is a worldwide disease that affects broilers in the first few weeks of age. It is a multifactorial disease and the exact agents causing the disease are not yet known, but different viruses and bacteria have been characterized (173). Experimental reproduction of MAS can be done by oral inoculation of 1-day-old broilers with homogenates obtained from digestive tissues of MAS affected chickens. The disease is characterized by weight gain depression with non-uniform growth, defective feathering and diarrhea with undigested food and watery content. Most lesions are present in the digestive organs, in particular in the small intestine. Because MAS mainly affects the intestine, it can be used as a model to study intestinal health and intestinal disturbances in young broilers.

Different broiler lines differ in their susceptibility for MAS as measured by a difference in growth retardation and the amount of lesions in the intestine after experimentally induced MAS. Also differences in the ratio of $CD4^+$: $CD8^+$ cells in the jejunum between those lines in infected as well as control conditions were found (171, 209). The basic mechanisms underlying the cause(s) of the differences in susceptibility for MAS are currently unknown. The identification of differentially expressed genes in susceptible and less susceptible chickens under control and MAS affected conditions

may lead to a better understanding of the cellular processes that determine susceptibility to MAS and maybe to other diseases.

The transcriptional response in the intestine of broilers is reported here after MAS induction and on the difference in gene expression and MAS susceptibility. Gene expression differences in the intestine were investigated using a cDNA microarray containing more than 3000 EST derived from a normalized and subtracted intestinal cDNA library (183). The findings were confirmed using a quantitative RT-PCR.

Materials and methods

Chickens

Two broiler lines, S (susceptible) and R (resistant), were used in the present study (Nutreco®, Boxmeer, The Netherlands).They were described earlier as B and D respectively (209). Sixty one-day old chicks of each line (S and R) were randomly divided into two groups of 30 chicks. One group was orally inoculated with 0.5 ml of the MAS-homogenate (homogenate C in tryptose buffered broth (172)) and the other was the control group, orally inoculated with 0.5 ml Dulbecco's phosphate buffered saline (PBS). Five chicks of each group were randomly chosen and sacrificed at 8 hr, day 1, 3, 5, 7 and 11 post inoculation (pi) and tissue samples were collected. Pieces of the jejunum were snap frozen in liquid nitrogen and kept at -70°C until further use. Adjacent parts of the jejunum were fixed in 4% formaldehyde and used for histopathology and immunohistochemistry. The study was approved by the institutional Animal Experiment Commission in accordance with the Dutch regulations on animal experimentation.

The same set-up, lines and groups, was used for a second animal experiment, although in that experiment three chicks of each group were sacrificed at day 1, 3 and 13 pi. The same tissues were sampled.

RNA Isolation

Pieces of the jejunum were crushed under liquid nitrogen. 50-100 mg tissues of the different chicks were used to isolate total RNA using TRIzol reagent (Invitrogen, Breda, The Netherlands) according to instructions of the manufacturer with an additional step. The homogenized tissue samples were resuspended in 1 ml of TRIzol Reagent using a syringe and 21 gauge needle and passing the lysate through 10 times. After homogenization, insoluble material was removed from the homogenate by centrifugation at 12,000 × g for 10 minutes at 4°C.

For the array hybridization pools of RNA were made in which equal amounts of RNA from five different chickens of the same line, condition and timepoint were present.

Hybridizing of the Microarray

The microarrays were constructed as described earlier and contained 3072 cDNAs spotted in duplicate (183). Before hybridization, the microarray was pre-hybridized in 5% SSC, 0.1% SDS and 1% BSA at 42°C for 30 minutes. To label the RNA, the MICROMAX TSA labeling and detection kit PerkinElmer, Wellesly, MAwas used. The TSA probe labeling and array hybridization were performed as described in the instruction manual with minor modifications. Biotin- and fluorescein-labelled cDNAs were generated from 5 μg of total RNA from the chicken jejunum pools per reaction. The cDNA synthesis time was increased to 3 hours at 42°C, as suggested (84). Post-hybridization washes were performed according to the manufacturer's recommendations. Hybridizations were performed in duplicate with the fluorophores reversed. After signal amplification the microarrays were dried and scanned in a GeneTAC2000 (PerkinElmer, Wellesly, MA). The image was processed (geneTAC software, c) and spots were located and integrated with the spotting file of the robot. Reports were created of total spot information and spot intensity ratio for subsequent data analyses.

Analysis of the Microarray Data

After background correction the data were presented in an M/A plot where $M=\log_2 R/G$ and $A=\log_2\sqrt{(R\times G)}$(46). An intensity-dependent normalization was performed using the lowess fit function in the statistical software package R (203). The normalization was done with a fraction of 0.2 on all data points.

A total of 48 microarrays were used in the first experiment. For each of the six timepoints, the following four comparisons were made:

broiler line S control vs. broiler line R control

broiler line S MAS vs. broiler line R MAS

broiler line S control vs. broiler line S MAS

broiler line R control vs. broiler line R MAS

For each of the comparisons two microarrays were used. Therefore for each cDNA four values were obtained, two for one slide and two for the dye-swap. Genes with two or more missing values were removed from further analysis. Missing values were possibly due to a bad signal to noise ratio. A gene was considered to be differentially

expressed when the mean value of the ratio was > 2 or < -2 and the cDNA was identified with significance analysis of microarrays (based on SAM (180)) with a False discovery rate < 2%. Because a ratio is expressed in a $\log_2$ scale, a ratio of > 2 or < -2 corresponds to a more than fourfold up- or downregulation respectively.

Sequencing and Sequence Analysis

Bacterial clones containing an insert representing a differentially expressed gene were sequenced. First a PCR was performed. One reaction of 50 µl contained: 5 µl of 10× ExTaq buffer(TaKaRa, Kyoto, Japan), 1 µl dNTP mixture[5] (2.5 mM each), 0.1 µl nested primer 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and nested primer 2 (5'-AGCGTGGTCGCGGCCGAGGT-3', 100 pmol/µl), 0.125 µl ExTaq[5] (5 units/µl), 43.6 µl sterilized distilled water and a bacterial clone from the library. The PCR was performed using a thermocycler programmed to conduct the following cycles: 2 min 95°C, 40× {45 sec 95°C, 45 sec 69°C, 120 sec 72°C}, 5 min 72°C. The PCR amplification products were purified using Sephadex G50 fine column filtration.

1 µl of the purified PCR product was sequenced using 10 pmol of nested primer 1 and 4 µl of ABI PRISM BigDye Terminator Cycle Sequencing Ready reaction in a total volume of 10 µl. The sequence reaction consisted of 2 min 96°C, 40× (10 sec 96°C, 4 min 60°C). Sequencing was performed on an ABI 3700 DNA sequencer. Sequence results were analyzed using SeqMan 5.00. Sequences were compared with the NCBI non redundant and the EST Gallus gallus database using blastn and blastx options (6). A hit was found with the blast search when the E-value was lower than 1E-5. For unknown chicken genes, the accession number of the highest hit with the Gallus gallus EST database is given and a description of the highest blastx hit. For known chicken genes the accession number is given.

Quantitative LightCycler real time PCR

For a reverse transcription 200 ng RNA was incubated at 70°C for 10 minutes with random hexamers (Promega, Leiden, The Netherlands) (0.5 µg). After 5 minutes on ice, the following was added: 5 µl 5× first strand buffer (Life Technologies, Rockville, MD, 2 µl 0.1 M DTT, 1 µl Superscript RNase H- reverse transcriptase7 (200 Units/µl), 1 µl RNAsin (40 Units/µl), 1 µl 2 mM dNTP mixture5, water to a final volume of 20 µl. The reaction was incubated for 50 min at 42°C. The reaction was inactivated by heating at 70°C for 10 min. Generated cDNA was stored at -20°C until use.

PCR amplification and analysis were achieved using a LightCycler instrument (Roche, Basel, Switzerland). For each primer combination the PCR reaction was optimized (174). The primers are shown in Table 1. The reaction mixture consisted of 1 µl cDNA (1:10 diluted), 1 µl of each primer (10 µM solution), 2 µl LightCycler FastStart DNA Master SYBR Green mix, $MgCl_2$ (4mM) in a total volume of 20 µl. All templates were amplified using the following LightCycler protocol: a preincubation for 10 minutes at 95°C; amplification for 40 cycles: (5 sec 95°C, 10 s annealing temperature, 15 s 72°C). Fluorescent data were acquired during each extension phase. After 40 cycles a melting curve was generated by heating the sample to 95°C followed by cooling down to 65 for 30 sec and slowly heating the samples at 0.2 °C/s to 96°C while the fluorescence was measured continuously. In each run, four standards of the gene of interest were included with appropriate dilutions of the cDNA, to determine the cDNA concentration in the samples. All RT-PCRs amplified a single product as determined by melting curve analysis.

Table 1. Sequences of primers used for LightCycler RT-PCR.

Genename/ homology	Forward primer	Reverse primer
Avian nephritis virus	ATTGCACAGTCAACTAATTTG	AAAGTTAGCCAATTCAAAATTA
Calbindin	CATGGATGGGAAGGAGC	GCTGCTGGCACCTAAAG
Gastrotropin	TAGTCACCGAGGTGGTG	GCTTTCCTCCAGAAATCTC
HES1	TCTTCCCAGGCTGTGAG	GGTCACCAGCTTGTTCTTC
Interferon-induced 6-16 protein	CGATCATGTCTGGTGAGGC	AGCACCTTCCTCCTTTG
Lysozyme G	CGGCTTCAGAGAAGATTG	GTACCGTTTGTCAACCTGC
Meprin	TTGCAGAATTCCATGATCTG	AGAAGGCTTGTCCTGATG
Pyrin	CCTGCACTGACCCTTG	GTGGCTCAGGGTCTTTC

Results

Differences between control and MAS induced chickens

All chickens inoculated with the MAS-homogenate developed growth retardation, which is the main clinical feature of MAS. A significant reduction in body weight gain relative to the controls was found in the susceptible chickens compared to the body

weight gain reduction in the resistant chickens after MAS induction (data not shown). A comparison of the gene expression in the chicken intestine was made in control and MAS induced chickens for the timepoints 8 hr, 1, 3, 5, 7 and 11 days pi of both broiler lines. The hybridization experiments showed different numbers of up- and downregulated genes after the MAS induction (Table 2). In general, more genes were found differentially expressed in the MAS susceptible broiler line compared to the resistant line. At day 1 pi most differentially expressed genes were found in both lines. The identity of the different up- and downregulated genes is shown in Table 3 and 4. To investigate if these genes are generally induced or repressed after a MAS induction, hybridizations were repeated with samples from animal experiment 2 where the same chicken lines were used. Samples were available from day 1, 3 and 13 pi. Comparing the two experiments the $\log_2$ (expression level MAS induced/expression level control) differed by 0.4 on average. Of the ratios, 69% differed less than 0.5, 18% between 0.5 and 1.0, 8% between 1.0 and 1.5 and only 5% differed more than 1.5. When a gene was upregulated more than fourfold in one experiment, it was also upregulated in the duplicate experiment, however not always more than fourfold.

Table 2. Number of differentially expressed genes[1] in Malabsorption affected chickens at different timepoints in different broiler lines.

	8 hr pi	day 1 pi	day 3 pi	day 5 pi	day 7 pi	day 11 pi
Number of genes upregulated after a mas infection						
Susceptible line	7	31	14	17	3	6
Resistant line	0	38	11	0	2	0
Number of genes downregulated after a mas infection						
Susceptible line	0	9	0	16	16	2
Resistant line	0	7	3	0	2	0

[1] A gene was declared differentially expressed when the mean value of the ratio was >2 or < -2 and the gene was identified with significance analysis of microarrays with a False discovery rate < 2%.

Differences between MAS susceptible and resistant broiler lines

The results of the comparison of infected versus control chickens indicated that there are clear gene expression differences between the two chicken lines used. Therefore samples from the two chicken lines were compared in control situation or in

Table 3. Chicken genes upregulated (U) or downregulated (D)[1] 4-fold after a malabsorption syndrome (MAS) induction.

chicken gene	description	Susceptible line	Resistant line
U73654.1	alcohol dehydrogenase	d1	d1
AF008592.1	inhibitor of apoptosis protein1	d1	
U00147	filamin	d1	
X52392.1	mitochondiral genome	d1 u5,11	
M31143.1	calbindin	d1,5,7,11	u1, d7
AJ236903.1	SGLT-1	d5	
AJ250337.1	cytochrome P450	d5,7	d1
M18421.1	apolipoprotein B	d5,7	
M18746.1	apolipoprotein AI	d5,7	
AF173612.1	18S rRNA	u8hr	u3,7
AF469049.1	caspase 6	u1	u1
U50339.1	galectin-3	u1	u1
AJ289779.1	angiopoietin 2C	u1,3,5	d1
L34554.1	stem cell antigen 2	u1,5	u1,3
D26311.1	unknown protein	u11	
AJ009799.1	ABC transporter protein	u3	d1
M10946.1	aldolase B	u3	u1,3
AF059262.1	cytidine deaminase	u5	u1
AJ307060.2	ovocalyxin-32	u5	
M27260.1	78 kDa glucose regulated protein	u5	
AY138247.1	p15INK4b tumor suppressor		d7
AJ006405.1	glutathion-dependent prostaglandin D synthase		u1

[1]U, D = genes up-or downregulated, respectively, at the sampling times indicated: 8 h, and 1, 3, 5, 7, and 11 d postinoculation.

MAS induced situation. In the control situation no significant differences between the two broiler lines were found except at day 11. Here 17 genes were identified which were expressed at least fourfold higher in the susceptible line at day 11 with a false discovery rate lower than 2% (Table 5). In the MAS induced situation at day 11 these genes differed not significantly between the two lines, most $\log_2$ ratios of these expression differences were between -1.0 and 1.0 with only two exceptions.

For the MAS affected situation, only significant differences between the two broiler lines were found at day 7 pi with a false discovery rate lower than 2% and at least a fourfold expression difference. However, at day 1 and 11 pi in the MAS affected

situation, genes were identified with a false discovery rate of 2.1 and 2.2% respectively, these genes were also considered to be significantly different in their expression levels. An overview of the genes differing between the two lines in the MAS induced situation is given in Table 6. All these genes lacked significant expression differences in the control situation with $\log_2$ ratios between -1.0 and 1.0.

Table 4. Chicken expressed sequence tags (EST) upregulated (U) or downregulated (D)[1] 4-fold after a malabsorption syndrome (MAS) induction.

chicken EST	homology	Susceptible line	Resistant line
BU123833	annexin A13	d1	
CD727681	pyrin	d1	
BU420110		d1,7	
BU124420	liver-expressed antibacterial peptide 2	d5	
BU217169	sucrase-isomaltase	d5	d1,3
BU292533	tubulointerstitial nephritis antigen-related protein	d5	
CD726841	zonadhesin	d5	
BU123839		d5	d1,3
BU124534	meprin	d5,7	
BU262937	angiotensin I converting enzyme	d5,7	
BU288276	mucin-2	d5,7	
BU480611		d5,7	u1
BU124511	Na+/glucose cotransporter	d7	
BU268030		d7	
BU464138		d7	
BU122834	pyrophosphatase/phosphodiesterase	u8hr	d1
BU122899	fatty acyl CoA hydrolase	u8hr,1	u1
BU467833	interferon-induced 6-16 protein	u8hr,1,3,5 d7	u1,3
-[2]	avian nephritis virus	u8hr,1,3,5,7 d11	u1,3 d7
BU138064	retionic acid and interferon inducible 58 kDa protein	u8hr,1,5	u1,3
BX258371	gastrotropin	u8hr,5 d1	d1
AI982261	ubiquitin-specific proteinase ISG43	u1	u1
BG712944	aminopeptidase	u1	
BU125579	cathepsin S	u1	

Table 4 (continued)

BU233187	zinc-binding protein	u1	u1
BU240951		u1	u1
BU255435	beta V spectrin	u1	u1
BU397837		u1	
BU492784	putative cell surface protein	u1	u1
BX273124	phosphofructokinase P	u1	
BU249257	unnamed protein product	u1	u1
-		u1	u1
BU296697	IFABP	u1, d5,7	u1
BU302098	Cl channel Ca activated	u1, d7	u1
BU410582	HES1	u1,11	u1,7
BU124153	Ca activated Cl channel 2	u1,11 d5,7	u1
AJ452523	mucin-like	u1,3	u1
BU118300	hensin	u1,3	u1
-	lymphocyte antigen	u1,3	u1
CD727020	interferon induced membrane protein	u1,3,5	u1,3
BU401950	lysozyme G	u1,3,5,7	u1,3
BU452240	14 kDa transmembrane protein	u1,3,5,7	u1,3
BU244292	transmembrane protein	u1,5	u1
BX271857	onzin	u1,5	u1,3
-		u11	
-	immunoresponsive gene 1	u11	
BU305240		u3	
BU130996	anterior gradient 2	u3,5	
BU378220		u5	u1

[1]U, D = EST up-or downregulated, respectively, at the sampling times indicated: 8 h, and 1, 3, 5, 7, and 11 d postinoculation.

[2] - = no EST in the database (august 2003).

Confirmation of gene expression differences

Array results can be influenced by each step of the complex assay, from array manufacturing to sample preparation and image analysis. Validation of expression differences is necessary with an alternate method. LightCycler RT-PCR was chosen for this validation, because it is quantitative, rapid and requires only small amounts of RNA.

Table 5. Genes expressed higher in the susceptible line compared to the resistant line in control situation at d11.

EST	Chicken gene/homology	log_2 ratio[1] in control situation	log_2 ratio[1] in MAS induced situation
BU123839	No homology	3.7	0.3
BU118300	hensin	3.7	1.7
BX271857	onzin	3.5	0.2
-[2]	Avian nephritis virus	3.3	-0.5
	Mitochondrial genome[3]	2.8	0.2
	cytochrome C oxidase subunit 1[3]	2.5	0.1
BU123664	No homology	2.3	-0.0
BU401950	lysozyme G	2.3	1.1
BU467833	interferon-induced 6-16 protein	2.3	0.2
	plasma membrane calcium pump[3]	2.2	-0.1
BU124318	immune associated nucleotide protein	2.2	-0.1
	Stem cell antigen 2[3]	2.2	0.0
-	lymphocyte antigen	2.2	0.1
	cytochrome C oxidase subunit III[3]	2.1	0.1
BX257981	No homology	2.1	0.3
-	No homology	2.0	0.6
-	No homology	2.0	0.9

[1] log_2 ratio is log_2 (expression level susceptible/expression level resistant).

[2] - = no expressed sequence tag in the database (August 2003).

[3] Chicken gene.

Eight differentially expressed genes were chosen for validation. They were differentially expressed in MAS induced chickens compared to control chickens and/ or were differentially expressed between the two chicken lines. Pools of RNA were tested for all time points. For the time point with the largest differences in gene expression, five individual animals were tested in the LightCycler. In contrast to the microarray, (relative) concentrations of mRNA are measured in the LightCycler RT-PCR, while the microarray detects expression differences. Therefore the average was taken of the LightCycler results of the individual animals and then converted to log_2 (infected/control). For all 8 genes tested the results with the pools of RNA were similar for the LightCycler and the microarray (Table 7). For seven of the eight genes tested, the differences between the two groups were significant for individual animals (P <

0.05). Only for gastrotropin at day 1 pi, the distribution of the results within the groups was rather spread.

Discussion

After a MAS induction a number of up- and downregulated genes could be expected. This is the first report of gene regulation in the chicken intestine as a response to a MAS induction. We studied two separate animal experiments in which chickens were induced with MAS in order to study the reproducibility of our findings and because no earlier reports about gene expression due to MAS were available. The results of the LightCycler RT-PCR were similar to the microarray data as expected, because both methods measure RNA levels. In the LightCycler concentrations are measured, while in a microarray experiment, relative expression differences are detected. The LightCycler is used in this study as well as other studies to validate microarray experiments (138). The LightCycler confirmed only for 71% of the genes the change in expression in another study (138), while we confirmed 100%. However, here we used glass slides with a two-color hybridization while the other study used high-density filters for the microarray. Also the threshold to detect differences was higher in our experiment, fourfold compared with twofold differences. Thus the genes described in this study are differentially expressed.

Gene expression differences after MAS induction

At day 1 pi most upregulated genes were found compared to the other time points investigated in both lines after a MAS induction. This could be due to the fact that heterophil infiltration in the mucosa starts already at 1 day pi. Another possible explanation of the amount of induced genes at day 1 is that MAS induction triggers epithelial apoptosis in the first days pi. Most downregulated genes were found at day 5 and 7 pi. During this time cystic crypts and villus atrophy were found. Therefore the amount of epithelial cells decreased. So possibly there is not a downregulation, but the percentage of epithelial specific genes in the total intestinal pool of RNA is decreased. It can also be that those genes are really downregulated in the cells but this needs to be further investigated.

Most identified induced or repressed genes are so far unknown in chicken or their function in the chicken intestine has not been determined. However, based on homology with known genes in other organisms a function can be predicted. The identified induced and repressed genes are related to different cellular functions. Examples of apoptosis related genes found after MAS induction are caspase 6, galectin-3, inhibitor of apoptosis protein 1 and angiopoietin 2C (103, 153, 208). Food

Table 6. Genes and expressed sequence tags (EST) differentially expressed in one of the broiler lines after a MAS induction.

EST	Chicken gene/ homology	day	line[1]	ratio MAS[2]	ratio control[3]
	SGLT-1[4]	1	S	2.2	-0.0
BU233187	zinc-binding protein	1	R	2.2	0.1
AJ295030	aldo-ketoreductase	1	R	2.3	0.8
BU307467	retinol-binding protein	1	R	2.4	-0.5
BX258371	gastrotropin	1	R	2.6	0.7
CD727681	pyrin	1	R	3.2	-0.3
-[5]	Avian nephritis virus	7	S	3.2	-0.2
BU401950	lysozyme G	7	S	2.7	0.7
BU296697	IFABP	7	R	2.2	0.3
BU268030	no homology	7	R	2.2	0.1
	cytochrome P450[4]	7	R	2.5	-0.2
	glutathione-dependent prostaglandin D synthase[4]	7	R	2.5	0.9
BU124534	meprin	7	R	2.7	-0.6
	Calbindin[4]	7/11	R	2.8/ 2.1	-0.4/-0.4
	cytidine deaminase[4]	11	S	2.0	0.3

[1] Broiler line with higher expression after MAS induction. S = susceptible; R= resistant.
[2] $\log_2$ ratio in MAS-induced situation.
[3] $\log_2$ ratio in control situation.
[4] Chicken gene.
[5] - = no expressed sequence tag in the database (August 2003).

absorption related genes are apolipoprotein A1 and B, sodium-glucose cotransporter, calbindin and aldolase B. Immune related genes are stem cell antigen 2 and interferon induced proteins. Also two different chloride channels were identified, as well as transmembrane proteins. Identified genes can also have other functions then mentioned, because the prediction is based on homology. Furthermore some genes have multiple functions, making it difficult to group these genes.

Interestingly, some upregulated genes were also found in a study of Marek's disease virus, like interferon inducible proteins and stem cell antigen 2 (124). In that *in vitro* study, gene expression following infection of chicken embryo fibroblasts with oncogenic Marek's disease virus was studied. Both genes have an immunological function and are involved in both MAS and Marek's disease. So possibly a part of the same immunological pathway is activated. Gene expression in the chicken intestine

Table 7. Results of LightCycler RT-PCR for 8 genes compared with the microarray results.

genename	day	array susceptible infected/ control	LightCycler susceptible infected/ control	array resistant infected/ control	LightCycler resistant infected/ control
anv	1	2.8	NA*	1.9	NA[1]
calbindin	7	-3.5	-2.7	-1.2	-3.2
gastrotropin	1	-2.7	-2.3	-2.3	-2.6
HES1	1	1.9	2.9	1.7	2.4
interferon-induced 6-16 protein	1	2.4	3.0	4.1	3.1
lysozyme G	1	3.4	11.2	3.8	13.4
meprin	7	-3.3	-3.4	-0.6	-1.3
pyrin	1	-4.2	-2.4	0.4	0.4

[1] All the control birds remained negative in the LightCycler experiment, therefore no ratio could be calculated.

after an *Eimeria* infection has been studied (119). The identity of the induced or repressed genes was different after a MAS induction compared to an Eimeria infection. However the infection models studied are quite different. *Eimeria* is a parasitic infection while the etiology of MAS is still unknown, but both viruses and bacteria are involved. Furthermore the source of the EST clones used for both studies differed, namely from a concanavalin-A activated splenic lymphocyte and a lipopolysaccharide activated macrophage cDNA library for the *Eimeria* study and from a MAS induced intestinal library in our study. It is not known if the same sequences are present on both microarrays.

Gene expression differences between broiler lines

In the susceptible line more up and down regulated genes after MAS induction were found. The susceptible line had a more severe weight gain reduction when compared to the resistant line after MAS induction. This weight gain reduction is related to the severity of the lesions in the jejunum. After histopathological examination of the jejunum of the MAS induced chickens it was found that the susceptible line developed more severe cystic crypts and villus atrophy compared to the resistant line. So probably the severity of the lesions in the jejunum due to MAS induction is reflected in the number of up and down regulated genes.

After a MAS induction some significant differences in gene expression were detectable between the two lines. Differences in gene expression between the two lines following a MAS induction were expected, because they differ in susceptibility for MAS in growth retardation and severity of intestinal lesions (209). The lower expression of aldo-ketoreductase, gastrotropin and intestinal fatty acid binding protein in the susceptible broiler line might cause poorer food absorption resulting in the growth retardation. The lower expression of calbindin might cause a deficiency in calcium absorption and therefore the weak bones observed in MAS affected broilers (182). Prostaglandins are important in healing mucosal injury and can downregulate the mucosal immune system (190), so it is interesting to establish the higher expression of a prostaglandin D synthase in the resistant broiler line. A gene of the avian nephritis virus is higher expressed in the susceptible line. This virus is probably present in the MAS homogenate, but is cleared faster from the resistant broiler line compared to the susceptible broiler line. The susceptible broiler line has also a longer upregulation of the antibacterial gene lysozyme G. The higher expression in the susceptible line of cytidine deaminase at day 11 might be an indication of recovery of severe lesions.

Differences in gene expression in control conditions between the broiler lines were detected on day 11. These differences were not found in MAS induced chickens. Possibly in the control situation there are differences between the two broiler lines in the development of the intestine. In the MAS induced situation the disease is dominant causing less regulation of genes involved in development. No differences in gene expression in control condition between the broiler lines were detected on earlier timepoints. This means that the gene expression levels at earlier timepoints are comparable in these two broiler lines in the control situation. Therefore all differences found in MAS induced situation at earlier time points are due to MAS and not to other differences. The identified gene expression differences at day 11 have a role in energy metabolism, immune system, or they are not yet characterized. Gene expression differences at day 11 might be important for the rate of recovery of the intestinal lesions which might also influence MAS susceptibility.

In summary, in this study up- and downregulated genes after a MAS induction were identified using a cDNA microarray. Also gene expression differences between two chicken lines differing in susceptibility for MAS were detectable. Most of the induced and repressed genes are currently unknown in chickens and they need to be characterized further before conclusions can be made about their function during MAS induction and susceptibility.

Chapter 4.

Gene expression responses to a *Salmonella* infection in the chicken intestine differ between lines

Saskia van Hemert
Arjan Hoekman
Mari Smits
Annemarie Rebel

Veterinary Immunology and Immunopathology (2006) 114: 247-258.

Abstract

Poultry products are an important source of *Salmonella enterica*. An effective way to reduce food poisoning due to *Salmonella* would be to breed chickens more resistant to *Salmonella*. Unfortunately host responses to *Salmonella* are complex with many factors involved. To learn more about responses to *Salmonella* in young chickens, a cDNA microarray analysis was performed to compare gene expression profiles between two chicken lines under control and *Salmonella* infected conditions. Newly hatched chickens were orally infected with *S. enterica* serovar Enteritidis. Since the intestine is the first barrier the bacteria encounter after oral inoculation, intestinal gene expression was investigated at different timepoints. Differences in gene expression between the two chicken lines were found in control as well as *Salmonella* infected conditions. In response to the *Salmonella* infection a fast growing chicken broiler line induced genes that affect T-cell activation, whereas in a slow growing broiler line genes involved in macrophage activation seemed to be more affected at day 1 post infection. At day 7 and 9 most gene expression differences between the two chicken lines were identified under control conditions, indicating a difference in the intestinal development between the two chicken lines which might be linked to the difference in *Salmonella* susceptibility. The findings in this study have lead to the identification of novel genes and possible cellular pathways which are host dependent.

Introduction

Salmonella enterica infections originating from poultry products are one of the most common causes of food poisoning in humans (137). The two serotypes that are the most frequently reported worldwide are *S. enterica* serovar Typhimurium and serovar Enteritidis. Incidences of reported salmonellosis have increased dramatically since 1980 and, at present, up to 3.7 million cases of salmonellosis are estimated to occur annually in the United States only (116). Human infection occurs in two major patterns; a systemic disease known as typhoid fever among others caused by *S. enterica* serovar Typhi and a gastrointestinal disease termed salmonellosis. The two most prevalent serovars causing salmonellosis are *S. enterica* serovar Typhimurium and serovar Enteritidis.

The outcome of an encounter between *Salmonella* and its host is dependent upon multiple factors including the host genetic background. To study the genetic factors involved in resistance to this pathogen, mouse models for *Salmonella* infection have widely been studied (113). Several genes and pathways have been identified that may influence the disease outcome (reviewed in (95, 151)). Animal models other than the mouse have been employed for the study of nontyphoidal *Salmonella* infections because the murine model is less suitable for the study of *Salmonella*-induced diarrhoea (157). Chicken has also been used to study *Salmonella* susceptibility, because in addition to its impact on human health, salmonellosis in young chickens results in economic losses for the poultry industry, amounting to approximately US$64 million to US$114 million annually in the United States(26). *S. enteritidis* can cause in chickens both systemic disease and symptom less intestinal infections. Salmonellosis in young chickens is characterized by severe clinical signs of systemic disease and by diarrhoea and dehydration with a high mortality rate. Host genetic factors clearly influence the epidemiology of *Salmonella* infection in chickens. It is important to clearly define resistance to *Salmonella* in chickens, as an inverse relationship between severity of caecal infection and colonisation in systemic organs has been suggested (91, 154). A general mechanism of resistance might apply to all serotypes of *Salmonella enterica* in chickens, as lines that had previously been shown to be resistant to *S. enterica* serovar Typhimurium were also found to be resistant to the serovars Gallinarum, Pullorum, and Enteritidis (28). Resistance to systemic salmonellosis in the chicken is a polygenic phenomenon as demonstrated in a number of studies (90, 96, 107, 108, 178). Studies in chickens have also revealed considerable differences between lines in levels of colonization of Salmonella of the gastrointestinal tract and responses to vaccination (97, 106). Polymorphisms in three candidate genes, NRAMP1, MHC Class 1 and IAP1 were found to be associated to the levels of *S.* serovar Enteritidis in the spleen of chicks infected at one-day of age, but played little role in *Salmonella* levels in the gastrointestinal tract (195). Susceptibility to *Salmonella*

caecal carrier state was investigated in chickens 1 to 6 weeks of age. A downregulation of IFN-γ was found in the caecal tonsils of susceptible chickens compared to the resistant animals (154).

So far, only candidate genes have been investigated for their possible role in the chicken response to a *Salmonella* infection, but a whole genome approach might give more insight into the genetic aspects of the host response. Gene expression array technology is a powerful tool that has already been used to expand the understanding of host-pathogen interactions. A number of reports have been published about host transcriptional responses to bacterial infection using gene arrays (reviewed in (150)). However, the effect of *Salmonella* on host gene expression using microarrays is so far not studied in chickens.

In this study the response on a *Salmonella* infection in relation to the genetic background of the host is studied. The gene expression profiles in the small intestines of a fast and a slow growing meat-type chicken line were compared in control and *Salmonella* infected conditions. We found differences in host gene expression as well as differences between the lines in host responses towards the *Salmonella* infection. Indeed it has been suggested that slow growing chickens are more resistant to *Salmonella* compared with fast growing ones (62). The gene expression differences found with the microarray were confirmed using quantitative reverse transcription (RT) -PCR.

Material and methods

Chickens

Two meat type chicken lines, fast growing line-A and slow growing line-B were used in the present study (Nutreco®, Boxmeer, The Netherlands). 80 one-day old chickens of each line (A and B) were randomly divided into 2 groups, 40 chickens each. After hatching, it was determined that birds were free of *Salmonella* by taking faecal samples, growing them overnight in buffered peptone water while shaking at 150 rpm and spread the samples on brilliant green agar + 100 ppm nalidixic acid for *Salmonella* determination (37°C, 18-24 hr).

Experimental infection

Salmonella enterica serovar Enteritidis phage type 4 (nalidixic acid resistant) was grown in buffered peptone water (BPW) overnight while shaking at 150 rpm. Of each chicken line, one group of 1-day old chickens was orally inoculated with 0.2 ml of the bacterial suspension containing 10^5 CFU *S. enterica* serovar Enteritidis. The CFU was determined by plating serial dilutions of the bacterial suspension on brilliant green agar plates with 100 ppm nalidixic acid. The control groups were inoculated with 0.2 ml saline. Five chickens of each group were randomly chosen and sacrificed at day 1, 3, 5,

7, 9, 11, 15 and 21 post infection (p.i.). Before euthanization the body weight of each chicken was measured. Pieces of the jejunum were snap frozen in liquid nitrogen and stored at -70°C until further analyses. The liver was removed and weighted and kept at 4°C until bacteriological examination. The study was approved by the institutional Animal Experiment Commission in accordance with the Dutch regulations on animal experimentation.

Bacteriological examination

For detection of *S.* serovar Enteritidis a cloacal swab was taken and after overnight enrichment by growing in BPW it was spread on brilliant green agar + 100 ppm nalidixic acid for *Salmonella* determination (37°C, 18-24 hr). One gram of liver of each bird was homogenized in 9 ml BPW, serial diluted in BPW, and plated onto brilliant green agar with nalidixic acid for quantitative *S.* serovar Enteritidis determination (37°C, 18-24 hr) by counting the colony forming units.

Statistics

A regression analysis over 8 timepoints was done with chicken line as experimental factor and log(CFU) as response variable. The same statistical analysis was performed on the weight of the chickens, so a regression analysis over timepoints with weight as a response variable. Calculations were performed in the statistical package Genstat 6. The weights of the control chickens from the two chicken lines were compared for each timepoint using the Student *t* test. A Student *t* test was also performed to compare the control chickens and the infected chickens within a chicken line. A P value < 0.05 was considered to be statistically different.

RNA Isolation

Pieces of the jejunum were crushed under liquid nitrogen. 50-100 mg tissues of the different chicks were used to isolate total RNA using TRIzol reagent (Invitrogen, Breda, the Netherlands), according to instructions of the manufacturer with an additional step. The homogenized tissue samples were resuspended in 1 ml of TRIzol Reagent using a syringe and 21 gauge needle and passing the lysate through 10 times. After homogenisation, insoluble material was removed from the homogenate by centrifugation at 12,000 × g for 10 minutes at 4°C. For the array hybridisation pools of RNA were made in which equal amounts of RNA from five different chickens of the same line, condition and timepoint were present.

Hybridising of the Microarray

The microarrays were constructed as described earlier (183). The microarrays contained 3072 cDNAs from a subtracted intestinal library and 1152 cDNAs from a

concanavalin A stimulated spleen library. All cDNAs were spotted in triplicate on each microarray. Before hybridisation, the microarray was pre-hybridised in 5% sodium chloride sodium citrate, 0.1% sodium dodecyl sulfate and 1% bovine serum albumin at 42°C for 30 minutes. To label the RNA, the MICROMAX TSA labelling and detection kit (PerkinElmer, Wellesley, MA) was used. The TSA probe labelling and array hybridisation were performed as described in the instruction manual with minor modifications. Biotin- and fluorescein-labelled cDNAs were generated from 5 μg of total RNA from the chicken jejunum pools per reaction. The cDNA synthesis time was increased to 3 hours at 42°C, as suggested (84). Post-hybridisation washes were performed according to the manufacturer's recommendations. Hybridisations were performed in duplicate with the fluorophores reversed. After signal amplification the microarrays were dried and scanned for Cy5 and Cy3 fluorescence in a Packard Bioscience BioChip Technologies apparatus. The image was processed with Genepix pro 5.0 (Genomic Solutions, Ann Arbor, MI) and spots were located and integrated with the spotting file of the robot used for spotting. Reports were created of total spot information and spot intensity ratio for subsequent data analyses.

Analysis of the Microarray Data

A total of 64 microarrays were used in this experiment. For each of the eight timepoints, the following four comparisons were made using pools of RNA from five different chickens:1- line-A (fast growing) control vs. line-B (slow growing) control, 2- line-A *Salmonella* vs. line-B *Salmonella*, 3- line-A control vs. line-A *Salmonella*, and 4- line-B control vs. line-B *Salmonella*. For each RNA six values were obtained, three for one slide and three for the dye-swap. Genes with two or more missing values were removed from further analysis. Missing values were possibly due to a bad signal to noise ratio. A gene was considered to be differentially expressed when the mean value of the ratio log2 (Cy5/Cy3) was > 1.58 or < -1.58 and the cDNA was identified with significance analysis of microarrays (based on SAM (180)) with a False discovery rate < 2%. Because the ratio was expressed in a log2 scale, a ratio of > 1.58 or < -1.58 corresponded to a more than threefold up- or downregulation respectively, which is the expression difference limit indicated by the manufacturer of the MICROMAX TSA labelling and detection kit (PerkinElmer, Wellesley, MA)Bacterial clones containing an insert representing a differentially expressed gene were sequenced and analysed using Seqman as described (184).

All the microarray information has been submitted into the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) website (http://www.ncbi.nlm.nih.gov/geo/). The accession number for the array is GPL2719. Each of the 64 slides has been submitted, and the accession numbers range from

GSM67074 to GSM67077, GSM67270 to GSM67321, and GSM67354 to GSM67361. The experiment series has been submitted as GSEI3066.

Quantitative LightCycler real time PCR

A quantitative PCR was performed as described previously (184). Briefly 200 ng of total RNA from the jejunum was reverse transcribed with random hexamers. Generated cDNA was stored at –20°C until use. PCR amplification and analysis for the ikaros transcription factor,the gene similar to mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase (GnT-IV), ZAP-70, apolipoprotein B and Cytochrome P450 was done with the described primers and conditions (Table 1). The reaction mixture consisted of 1 µl cDNA (1:10 diluted), 1 µl of each primer (10 µM solution), 2 µl LightCycler FastStart DNA Master SYBR Green mix, 4 mM $MgCl_2$ in a total volume of 20 µl. All templates were amplified with a preincubation for 10 minutes at 95°C followed by amplification for 40 cycles: (5 sec 95°C, 10 s annealing temperature, 15 s 72°C).

In each run, four standards of the gene of interest were included with appropriate dilutions of the DNA, to determine the cDNA concentration in the samples. All RT-PCRs amplified a single product as determined by melting curve analysis.

Table 1. Primers and RT-PCR conditions for different genes.

Gene	Primers for RT-PCR	$MgCl_2$ concentration	Annealing temperature
Ikaros	for: 5'-ATGTGCGGAGGATTTACGAA-3' rev: 5'- TTTGCCAACCGAGTGAGTC -3'	4 mM	58 ºC
GnT-IV	for: 5'-CATCGTTGTCAAGCAGAAT -3' rev: 5'- TTAAAGATGTGGAAACCTC -3'	5 mM	56 ºC
ZAP-70	for: 5'- TGGCCACATCGATCTGCTTCT -3' rev: 5'- GACGGGCTGATTTTCTACCTG -3'	5 mM	63 ºC
Apolipo-protein B	for: 5'- GAACTAACACGCGCGCTAATT -3' rev: 5'- TTCAGGTCTTCATGCGCTTCT-3'	3 mM	59 ºC
Cytochrome P450	for: 5'- CTTTGGGACATGCCTGGAA-3' rev: 5'- GCTGCCTGCCATCGTAAATC-3'	3 mM	59 ºC

Results

Bacteriological examination and body weight

In all the animals inoculated with *Salmonella enterica* serovar Enteritidis the *Salmonella* was detected in the cloacal swap at all time points analysed by bacterial platings. In contrast, in none of the control animal *S. enterica* serovar Enteritidis was

detected. The number of *S. enterica* serovar Enteritidis found in the liver of chickens from the fast growing (A) and slow growing (B) line is presented in figure 1. Analysis across the collected time points showed that line-A had higher amounts of CFU in the liver than line-B, although this difference was just above the statistical treshold of 0.05 (P<0.056). Regression analysis revealed that in line-A the (log)CFU increased till day 7 after which the CFU decreased while in line-B the amount of CFU decreased from day 1. The (log)CFU are quadratic decreasing in time (P= 0.02) for line-A and linearly decreasing (P=0.004) for line-B. So when the CFU data from the experiment were used to make a model, the factor for the decrease in time was different for both lines. This implies that the kinetics of the infection were different for the two lines.

Body weight differences were not detected in the control situation, between the two chicken lines till day 9. From day 11 onwards, the chickens from line-A were heavier than line-B (P<0.05). In figure 2 is shown that the chickens from line-A had a higher weight gain depression after *Salmonella* infection compared to the chickens from line-B, as was shown with regression analysis across the time points (P = 0.007). For line-A the infected chickens were significantly lighter (P<0.05) than their control counterparts from day 7 till day 21 while the infected chickens from line-B were significantly lighter than their control counterparts only at day 7 and 15.

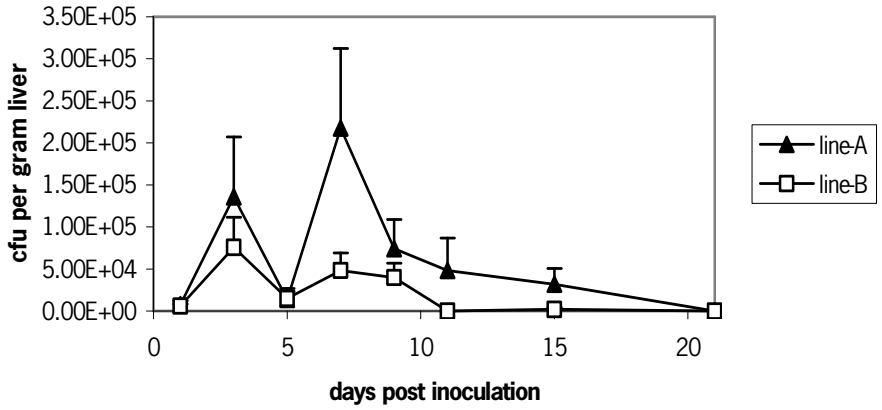

Figure 4.1. Number of CFU of *S. enteritidis* in the liver of chickens from the two chicken lines (n=5).

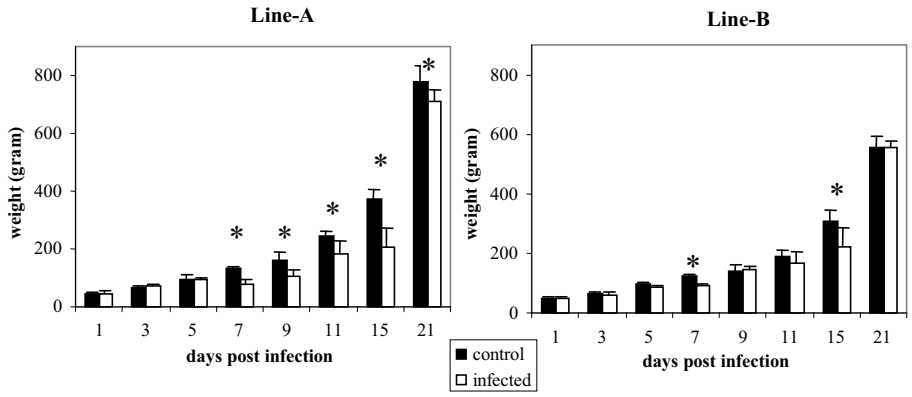

Figure 4.2. Mean body weight of two broiler lines under control conditions or after infection with 10^5 *S.* enteritidis (n=5). * indicates a significant difference ($p<0.05$) between the control and the *Salmonella* infected chickens.

Gene expression differences between the chicken lines

Changes in mRNA expression in the jejunum in response to infection with *Salmonella* were compared in both chicken lines on 8 different timepoints. Genes included for further analysis needed to meet the following two criteria: their expression was altered more than threefold due to the *Salmonella* infection in only one of the two chicken lines and their expression differed more than threefold between the chicken lines either in the control situation, or the *Salmonella* infected situation. Most genes differing between the two chicken lines after the *Salmonella* infection were found at day 1. In the control situation most differences between the chicken lines were found at day 9. After day 15 only a few differentially expressed genes were identified between the chicken lines in control and *Salmonella* infected chickens. Because most differences were found at day 1, 7 and 9 these timepoints will be described in detail**.**

Gene expression response at day 1

All the described genes were up- or downregulated in response to the *Salmonella* infection in only one of the chicken lines (A or B). Furthermore they differed in expression levels between the two chicken lines under control conditions or after the *Salmonella* infection.

In line-A 13 upregulated and two downregulated genes were identified after the *Salmonella* infection (Table 2, A_{sal}/A_{cont}). These 15 genes were equally expressed in

both chicken lines under control conditions (Table 2, A_{cont}/B_{cont}). None of these 15 genes were regulated in line-B due to Salmonella infection (Table 2, B_{cont}/B_{sal}). Because these genes were regulated in line-A after infection and not regulated in line-B, expression differences of these 15 genes between the two infected chicken lines were found (Table 2, B_{sal}/A_{sal}).

In line-B three genes were found to be upregulated and six genes were downregulated in response to *Salmonella* (Table 2, B_{sal}/B_{cont}). These 9 genes were not induced in line-A after Salmonella infection (Table 2, A_{cont}/A_{sal}). Two of these genes were equally expressed in both chicken lines under control conditions, the other seven genes differed in the control situation between the two lines (Table 2, B_{cont}/A_{cont}), The two genes that were upregulated in line-B due to the *Salmonella* infection were not regulated in line-A, therefore expression differences of these two genes were also found between the two infected chicken lines (Table 2, B_{sal}/A_{sal}). The 7 genes that differed in control situation were induced by Salmonella infection in line-B and not in line-A, due to their expression differences in control conditions no expression differences were found when Salmonella infected tissue of line-B was compared with line-A.

Gene expression at day 7 and 9

Most differences in expression levels between the two chicken lines in the control situation were detected at day 9 post infection. At this timepoint 34 genes were identified with different expression levels under control conditions between the two lines. Furthermore at day 9 these genes were regulated in response to *Salmonella* only in line-B (data not shown). Due to the higher expression levels under control conditions in line-B compared to control line-A and the downregulation of these genes after the *Salmonella* infection in line-B and not in line-A, there were no expression differences found for these genes between the two infected lines. Interestingly, 28 out of these 34 genes also differed at day 7 under control condition between the two chicken lines (Table 3). However at day 7 no regulation of more than threefold was found in either chicken line in response to the *Salmonella* infection.

Strikingly the following 9 genes differed in expression levels between the two chicken lines at day 7 and 9 in control conditions as well as at day 1 in *Salmonella* infected conditions: similar to mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, ikaros transcription factor, ZAP-70, CDH-1D and five uncharacterised genes. The expression differences between the chicken lines at day 1 were not detected under control condition as was shown for day 7 and 9. At the other timepoints investigated (day 3, 5, 11, 15 and 21) no expression differences of more than threefold were found for these 34 genes.

Table 2. Genes at day 1 with more than threefold expression differences due to the *Salmonella* infection in only one of the two chicken lines (A or B) and expression differences between the chicken lines either in the control situation (cont), or the *Salmonella* infected situation (sal).

Accession no.	Gene description	Human homologue	A_{sal}/A_{cont} [a]	B_{sal}/B_{cont} [a]	B_{cont}/A_{cont} [b]	B_{sal}/A_{sal} [b]
Regulated in chicken line-A						
NM_001012824.1	similar to mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme A; UDP-N-acetylglucosamine:alpha1 (GnT-IV)	MGAT4A	+ 2.11	0	0	- 3.23
Y11833.1	GGIKTRF G.gallus mRNA for Ikaros transcription factor	ZNFN1A1	+ 2.01	0	0	- 3.61
XM_418206.1	similar to Tyrosine-protein kinase ZAP-70 (70 kDa zeta-associated protein) (Syk-related tyrosine kinase)	ZAP70	+ 1.61	0	0	- 2.99
AJ719433.1	mRNA for hypothetical protein, clone 2e14	PTPN6	+ 1.66	0	0	- 3.69
CR387311.1	finished cDNA, clone ChEST351c21		+ 1.78	0	0	- 3.38
DN828706	expressed sequence tag (Chr:2 62779859- 62780676)		+ 1.74	0	0	- 3.69
DN828699	expressed sequence tag (Chr:6 16831318-16831844)		+ 1.97	0	0	- 2.82
BU227174	expressed sequence tag (Chr:2 13503808 -13504612)		+ 1.92	0	0	- 2.86
DN828707	expressed sequence tag (Chr:7 650389- 651174)		+ 2.59	0	0	- 3.47
DN828697	expressed sequence tag (Chr:2 116417100 –116437880)		+ 1.62	0	0	- 2.89
AF421549	CDH1-D		+ 2.22	0	0	- 3.58
CR389073.1	finished cDNA, clone ChEST347g18		+ 1.65	0	0	- 2.56
XM_421959.1	PREDICTED: similar to inducible T-cell co-stimulator	ICOS	+ 1.63	0	0	- 2.84
M18421	apoB mRNA encoding apolipoprotein	APOB	-1.62	NA	0	+1.74
NM_001001751.1	cytochrome P450 A 37 (CYP3A37)	CYP3A	-1.62	0	0	+1.69

Table 2 (continued)

Regulated in chicken line-B						
CD726841.1	expressed sequence tag (Chr: 9 12560405-12564673)		0	+ 1.63	0	+2.14
XM_422715	PREDICTED: similar to Fc fragment of IgG binding protein; IgG Fc binding protein	FCGBP	0	+ 1.58	0	+2.64
XM_421662.1	PREDICTED:similar to Interferon-induced protein with tetratricopeptide repeats 5 (IFIT-5) (Retinoic acid- and interferon-inducible 58 kDa protein)	IFIT5	0	+ 2.03	- 1.63	NA
XM_417585.1	PREDICTED: similar to tumor necrosis factor receptor superfamily, member 18 isoform 3 precursor; glucocorticoid-induced TNFR-related protein; activation-inducible TNFR family receptor; TNF receptor superfamily activation-inducible protein	TNFRSF18	0	-1.66	+1.81	0
XM_423002.1	PREDICTED: similar to Rho GTPase-activating protein; brain-specific Rho GTP-ase-activating protein; rac GTPase activating protein; GAB-associated CDC42; RhoGAP involved in the -catenin-N-cadherin and NMDA receptor signaling		0	-1.68	+1.82	0
DN828701	expressed sequence tag (Chr:2 80621800 -80622343)		0	-1.78	+1.96	0
BU457068.1	cDNA clone ChEST200c16		0	-1.73	+1.99	0
XM_425603.1	PREDICTED: Gallus gallus similar to ORF2		0	-1.9	+2.08	0
XM_416085.1	PREDICTED:similar to Carboxypeptidase M precursor	CPM	0	-2.02	+2.34	0

[a] 2log(infected/control). A negative value means a downregulation, a positive value a upregulation and a 0 that the expression differences were less than threefold.

[b] 2log(line-B/line-A). A negative value means higher expression in line-A, a positive value means lower expression in line-A and a 0 means that the expression differences between the chicken lines were less than threefold. All values has a p-value <0.02. NA is not available.

Confirmation of the microarray data

Validation of the microarray data was done with LightCycler RT-PCR, because it is quantitative, rapid and requires only small amounts of RNA. ZAP-70, the ikaros transcription factor and the gene similar to mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase (GnT-IV) were tested at day 1, 7 and 9. Unfortunately at day 1 no expression differences could be found with the LightCycler for these genes, because the expression levels were below our detection limit. At day 7 and 9 the expression levels were higher in all groups and expression could be detected, except for ZAP-70 at day 7.

To confirm our microarray data at day 1 apolipoprotein B and cytochrome P450 were tested with the LightCycler RT-PCR. These genes had higher expression levels compared to the ZAP-70, the ikaros transcription factor and GnT-IV, so expression could be detected at day 1 for apolipoprotein B and cytochrome P450. To further confirm the microarray data we repeated the microarray hybridisations for day 1, with new prepared RNA from the same intestine. These hybridisations gave similar results for all the described genes (results not shown).

With the LightCycler RT relative concentrations of mRNA are measured, while the microarray detects expression ratios. Therefore the expression ratios between the two chicken lines were calculated for the control animals and the *Salmonella* infected animals. Since the LightCycler RT-PCR is quantitative and individual animals were tested, the cut off value for significance is lower when compared to the micro-array. More than two-fold expression differences in the RT-PCR were significant for all genes using a student T-test. For all the tested genes the results of the microarray were confirmed with the RT-PCR (Table 4). For apolipoprotein B and cytochrome P450 no expression differences between the two chicken lines were found under control conditions, while after the *Salmonella* infection higher expression levels were detected in chicken line-B. For ZAP-70, the ikaros transcription factor and GnT-IV at day 7 and 9.

The control animals of chicken line-B had higher expression levels for these genes compared to line-A. After the salmonella infection no expression differences between the two chicken lines were found (Table 4).

Table 3. Genes with different expression levels between the two chicken lines (A or B) in the control situation at day 7 and 9.

accession no.	gene name	Human homologue	day 7[a]		day 9[a]	
			B_{cont}/A_{cont}	B_{sal}/A_{sal}	B_{cont}/A_{cont}	B_{sal}/A_{sal}
NM_001012824.1	similar to mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme A; UDP-N-acetylglucosamine:alpha1 (GnT-IV)	MGAT4A	1.95	0	2.75	0
Y11833.1|	GGIKTRF G.gallus mRNA for Ikaros transcription factor	ZNFN1A1	2.06	0	2.50	0
XM_418206.1	similar to Tyrosine-protein kinase ZAP-70 (70 kDa zeta-associated protein) (Syk-related tyrosine kinase)	ZAP70	2.33	0	2.73	0
AF421549	CDH1-D		2.23	0	2.60	0
AJ719433.1	mRNA for hypothetical protein, clone 2e14	PTPN6	2.23	0	2.73	0
CR387311.1	finished cDNA, clone ChEST351c21		2.33	0	2.12	0
DN828706	expressed sequence tag (Chr:2 62779859- 62780676)		2.59	0	2.35	0
DN828699	expressed sequence tag (Chr:6 16831318-16831844)		2.07	0	2.29	0
BU227174	expressed sequence tag (Chr:2 13503808 -13504612)		2.25	0	2.45	0
XM_417797.1	PREDICTED: similar to protein tyrosine phosphatase 4a2	PTP4A2	1.78	0	2.03	0
NM_001012914.1	signal transducer and activator of transcription 4 (STAT4)	STAT4	2.00	0	2.28	0
XM_419701.1	PREDICTED: similar to T-cell activation Rho GTPase-activating protein isoform b	TAGAP	2.30	0	2.19	0
NM_001006289.1	similar to 14-3-3 protein beta/alpha (Protein kinase C inhibitor protein-1) (KCIP-1) (Protein 1054)	YWHAB	2.27	0	1.75	0
NM_204417.1	protein tyrosine phosphatase, receptor type, C (PTPRC)	PTPRC	2.27	0	2.23	0
XM_420925	PREDICTED: similar to interferon-induced membrane protein Leu-13/9-27	IFITM3	3.10	0	1.67	0
AJ725129	riken1 cDNA clone 29g19s4, mRNA sequence		2.13	0	2.51	0
AJ719476.1	mRNA for hypothetical protein, clone 2k22	SS18	2.18	0	1.76	0

Table 3 (continued)

AJ719498.1	mRNA for hypothetical protein, clone 2n23	2.48	0	2.27	0
AJ443170	dkfz426 cDNA clone 33p14r1, mRNA sequence	2.55	0	1.92	0
DN828698	expressed sequence tag (Chr:1 45555037-45555282)	2.07	0	1.73	0
BU216613	expressed sequence tag (Chr:1 68764871 -68765523)	2.46	0	1.84	0
DN828705	expressed sequence tag (Chr:1 96252022 -96252307)	1.64	0	2.44	0
DN828703	expressed sequence tag (Chr:2 90373281 -90373444)	2.13	0	2.21	0
DN828702	expressed sequence tag (Chr:2 119819797-119820008)	2.18	0	1.67	0
DN828704	expressed sequence tag (Chr:3 89463019 -89463394)	2.25	0	1.74	0
DN828700	expressed sequence tag (Chr:5 24855130 –24855627)	2.12	0	1.77	0
DN828696	expressed sequence tag (Chr:23 908128- 908579)	2.52	0	2.07	0
BU128188	expressed sequence tag (Un 143410092-143411009)	3.17	0	1.82	0

[a] 2log(line-B/line-A). A positive value > 1.58 means a more than threefold higher expression in line-B, a negative value < -1.58 means a more than threefold higher expression in line-A and a 0 means that the expression differences between the chicken lines were less than threefold. All values has a p-value <0.02. Differences in control (contr.) and *Salmonella* infected (inf.) conditions are given.

Table 4. Ratio of the expression levels (line-B/line-A) found with the LightCycler RT-PCR and the microarray for 5 different genes.

		control		salmonella	
Gene name		microarray	RT-PCR	microarray	RT-PCR
Apolipoprotein B	day 1	1.2	1.9	**3.1**	**4.7**
Cytochrome P450	day 1	0.5	0.9	**3.3**	**3.2**
GnT-IV	day 7	**3.9**	**4.2**	1.6	0.6
	day 9	**6.7**	**2.6**	0.5	0.9
Ikaros transcription factor	day 7	**4.2**	**2.4**	1.2	0.7
	day 9	**5.7**	**2.2**	0.6	1.0
ZAP-70	day 7	5.0	n.d.	1.6	n.d.
	day 9	**6.6**	**3.3**	0.6	1.0

Bold are differences in expression levels found with the microarray as well as the RT-PCR. n.d. is not detectable.

Discussion

In this study host gene expression responses to a *Salmonella* infection were studied in two broiler lines, measuring mRNA levels with a cDNA microarray. It should be remembered that the microarray technology only gives information about steady-state levels of mRNA. Differences in the amount of mRNA will not always result in differences in the amount of active proteins, however mRNA levels can give an indication to the amount of proteins and they are easily measured. The results of the microarray were confirmed with a quantitative RT-PCR. Unfortunately at day 1 with the RT-PCR no expression could be detected for ZAP-70, the ikaros transcription factor and the gene similar to mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase (GnT-IV). One reason that we were not able to detect these genes could be the low level of expression. For the microarray 5 μg of total RNA is used for the generation of cDNA, while for the RT-PCR only 1 ng of total RNA was used as input, so our used microarray can detected lower expression levels compared to the performed LightCycler RT-PCR. At day 7 and 9 the results with the microarray and the RT-PCR were comparable as were the results at day 1 for apolipoprotein B and cytochrome P450. To confirm the micro-array data, RNA of day 1 old birds was re-isolated from jejunum adjacent from the part used before that was stored in the -70. With this RNA the microarray experiment was repeated. From this experiment all genes were confirmed. Again at least 4 of the 6 spots of one gene had intensities for both fluorphores above the background. Conformation of our microarray data with the

RT-PCR was also showed in earlier studies (184), suggesting that our findings with the microarray are highly reliable.

It should also be mentioned that the cDNA libraries used to construct the microarray contain a limited number of genes, so it is reasonable to assume that we have missed genes that are involved the response to a *Salmonella* infection. However, the used cDNA libraries are subtracted, normalized and tissue-specific, increasing the chance that the array contained genes relevant for intestinal health. The role the proteins encoded by the identified genes in the response to *Salmonella* is speculative. For the expressed sequence tags no further gene prediction was found with the Ensembl Chicken Genome Browser (www.ensembl.org). Also for some genes no function is known, like the finished cDNA clones. Other genes are studied in other organisms than chicken. Furthermore a gene can be bi- or multifunctional. Nevertheless we can hypothesize for a number of genes about their role in *Salmonella* response and their differences in regulation due to host genetic background.

Investigation of the gene expression responses to *Salmonella* was started at day 1 post infection, because early timepoints will provide more knowledge about the early events instead of showing the consequences of infection or inflammation. At day 1 distinct differences in gene expression were found comparing the two chicken lines. Differences in response to the *Salmonella* infection were found as well as differences in the control situation of age matched chickens. In chicken line-A a number of uncharacterised genes was upregulated in response to the *Salmonella* infection as well as some known genes. The known genes indicates that T-cell activation or T-cell maturation differ between the two lines as a response to a Salmonella infection.. The Ikaros transcription factor is up-regulated in line A and has an important function in T-cell development (94). ZAP-70 is also up-regulated in line A and plays a fundamental role in the initial step of the T-cell receptor signal transduction (38), and probably also plays an important role in growth and differentiation in several tissues including the intestine (75). CDH1-D, the third identified gene, has a role in the regulation of the cell cycle (192). The gene similar to mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase (GnT-IV) was also upregulated at day 1 in line-A. GnT-IV is upregulated during differentiation and development and highly expressed in leukocytes and T-cell associated lymphoid tissues, like the small intestine and is one of the key glycosyltransferases regulating the formation of highly branched complex type *N*-glycans on glycoproteins (207). The inducible T-cell co-stimulator is not expressed on naïve T-cells, but requires the activation of T-cells via the T-cell receptor (147).These upregulated genes in line-A at day 1 pi suggest that T-cells are in another direction activated, maturated or more activated in chicken line-A at day 1 due to the *Salmonella* infection compared to line-B. It is in line with other findings, showing that

an oral *S. enterica* serovar Enteritidis infection increased the number of T-cells in the intestine, suggesting that a *Salmonella* infection either stimulated gut-associated T-cells to expand or recruite more T-cells to the mucosal tissues (163). Furthermore expression of the CXC chemokines IL-8 and K60 was upregulated in the jejunum of *Salmonella* serovar Typhimurium infected chicken early after the infection (196). As CXC chemokines are chemoattractant for polymorphonuclear cells and naïve T-cells, this further confirms the role of T-cell activation in the early response to a *Salmonella* infection in chicken line-A while in chicken line-B other processes might be more dominant.

Cytochrome P450 and apolipoprotein B were downregulated at day 1 in line-A and not in line-B. They were also downregulated in a chicken line when malabsorption syndrome was studied (184), a model for intestinal disturbances in young chickens. Downregulation of apolipoprotein B and cytochrome P450 in intestinal epithelium was also shown in response to proinflammatory cytokines (21, 189) . So the downregulation of apolipoprotein B and cytochrome P450 might be a response to disturbances in the intestine.

In contrast to the *Salmonella* infected chickens from line-A the infected chickens from line-B did not upregulate genes involved in T-cell activation in response to the infection. On the contrary at day 1 post infection a TNF receptor was downregulated in line-B in response to *Salmonella* while expression of this gene is strongly increased upon T-cell activation (131). In the control situation this gene also differed in expression between the two chicken lines with higher expression in line-B. $CD4^+$ cells have a higher expression of this TNF receptor compared to $CD8^+$ cells (131), so possibly line-B has more $CD4^+$ cells in the jejunum. However, the chicken lines might also differ in the amount of macrophages, as expression of the TNF receptor is also shown in macrophages (164).This latter suggestion is supported by carboxypeptidase M, a macrophage differentiation marker (144), which is also higher expressed in line-B in the control situation compared to line-A. After the *Salmonella* infection carboxypeptidase M is downregulated in line-B as is the TNF receptor, so possibly chicken line-B has a better macrophage activation compared to line-A at day 1 post infection.

At day 9 post infection gene expression differences in the control situation were detected between the two chicken lines. Most of these differences were also detected at day 7, indicating the robustness of our results. As line-A grows faster than line-B it is not surprising to find differences in the control situation at the intestinal level. From day 11 onwards the weights of the control chickens from both lines differ significantly.

The differences in gene expression at day 7 and 9 in the control situation might reflect a difference in the development of the intestine of the young chickens, as the expression of these genes was higher at day 7 and 9 compared to day 1, which was indicated by the quantitative RT-PCR results. This suggests that the expression of these genes increases in time due to the development of the gut or to differences in stimulation of the immune system by microbes developing the gut flora in the young animals (71). It is known that the morphology of the small intestine changes rapidly after hatch (58), but the early changes in intestinal morphology was not studied for chickens differing in growth rate. However, it is known that genetic selection on growth rate has effects on the intestinal structure of chickens of four weeks old (168). Nine of the genes found at day 7 and 9 in the control situation also showed expression differences at day 1 after *Salmonella* infection. Five of these genes are uncharacterised, but the remaining four have a function in T-cell activation which would suggest that the development of the immune system in the gut differs between the two lines.

The differences in gene expression responses found between the two chicken lines might be related to a difference in *Salmonella* susceptibility. The chickens from the fast growing line-A had more CFU in the liver and the clearance was slower compared to the slow growing line-B, with a different kinetic profile of clearance of the *Salmonella* from the liver. The chickens from line-A also had more weight gain depression following the *Salmonella enterica* serovar Enteritidis. The difference in liver clearance and weight gain depression over time showed that line-A was more susceptible to *Salmonella* compared to line-B. This is in accordance with earlier studies, where was suggested that genetic selection towards enhanced performance traits has negatively influenced the immune system (34). Also meat type chickens selected for a high body weight showed a lower natural resistance to *Salmonella* compared to Old Dutch Breeds (92).

This study has given more insight into the molecular host response to *Salmonella*. Furthermore this study has revealed differences in gene expression responses to a *Salmonella* infection in different chicken lines. Gene expression indicated that T-cells are more activated in one chicken line in response to the *Salmonella* infection, while the other chicken line seemed to have an increased macrophage activation at day 1 post infection. Marked expression differences were also found for multiple uncharacterized genes. Although the precise function for most of the identified genes is yet unclear, the found differences between the two chicken lines might be related to the differences in *Salmonella* susceptibility found in these two lines. Further studies using other chicken lines and chicken lines genetically selected for *Salmonella* resistance will be valuable

to test this hypothesis. Also further studies will be needed in understanding the function of the identified genes and identification of the relevant pathways.

Acknowledgements

We thank our animal care takers for their technical support.

This study was financially supported by the Animal Sciences Group of Wageningen UR, the Netherlands.

Chapter 5.

Early host gene expression responses to a *Salmonella* infection in the intestine of chickens with different genetic background examined with cDNA and oligonucleotide microarrays

Saskia van Hemert
Arjan Hoekman
Mari Smits
Annemarie Rebel

Comparative Biochemistry and Physiology D (2006) 1: 292-299

Abstract

So far the responses of chickens to *Salmonella* have not been studied *in vivo* on a whole genome-wide scale. Furthermore, the influence of the host genetic background on gene expression responses is unknown. In this study gene expression profiles in the chicken (*Gallus gallus*) intestine of two genetically different chicken lines were compared, 24 hours after a *Salmonella* enteritidis inoculation in 1-day-old chicks. The two chicken lines differed in the severity of the systemic infection. For gene expression profiles, a whole genome oligonucleotide array and a cDNA microarray were used to compare both platforms. Genes upregulated in both chicken lines after the *Salmonella* infection had a function in the innate immune system or in wound healing. Genes regulated after the *Salmonella* infection in one chicken line encoded proteins involved in inflammation, or with unknown functions. In the other chicken line upregulated genes encoded proteins involved in acute phase response, the fibrinogen system, actin polymerisation, or with unknown functions. Some of the host gene responses found in this study are not described before as response to a bacterial infection in the intestine. The two chicken lines reacted with different intestinal gene responses to the *Salmonella* infection, implying that it is important to use chickens with different genetic background to study gene expression responses.

Introduction

Salmonella enterica is one of the most common causes of food poisoning in humans, mostly caused by poultry products infected by *S. enterica* serovars Typhimurium or Enteritidis (137). Following oral ingestion, *Salmonella* colonize the intestines and invade the intestinal mucosa. In addition to the enteric disease in humans *Salmonella* serovars Typhimurium and Enteritidis are also capable of causing severe systemic disease in newly hatched chicks and in birds under extreme stress conditions. The infection seldom causes mortality in birds more than 1 month old (176). In young chickens infection with *Salmonella* leads to diarrhea and intestinal lesions and to an influx of heterophils into the gut accompanied by inflammation and damage to villi (13). Heterophils are the avian equivalent of mammalian neutrophils and play a key role in protecting chickens from the development of systemic disease following infection with *Salmonella* serovar Enteritidis by largely restricting the bacteria to the gut (89).

Host gene expression responses to a *Salmonella* infection have widely been studied. One of the methods to investigate gene expression responses is the use of microarrays that allow the analysis of the expression of a large number of genes in a single experiment. Indeed microarrays have been used to study gene expression responses to *Salmonella* (reviewed in (150)). With this approach it was found that in human epithelial cells cultures *Salmonella* typhimurium induce a classical proinflammatory gene expression pathway with upregulation of several cytokines, kinases and transcription factors (48, 212). Also in human macrophages *Salmonella* induces a set of gene products, many of which are proinflammatory (43). As in other organisms, *Salmonella* is capable to alter gene expression levels in chickens; however, mainly cytokine levels have been studied in response to *Salmonella* infections. In the intestine and liver interleukin (IL) 8, K60 (a CXC chemokine), macrophage inflammatory protein 1 β, and IL-1β levels were upregulated in response to *Salmonella* (196), suggesting inflammation in those tissues. Also in heterophils, upregulation of pro-inflammatory cytokines IL-6, IL-8 and IL18 was detected after exposure to *Salmonella* as well as a downregulation of transforming growth factor-β4, an anti-inflammatory cytokine (178), indicating inflammatory processes to be important after *Salmonella* exposure. The role of IFNγ in the response to *Salmonella* is less clear, up and downregulation as well as no regulation in response to *Salmonella* has been reported (81, 154, 166). In addition to the cytokines also cationic liver-expressed

antimicrobial peptide 2 was shown to be upregulated in the chicken intestine and liver in response to *Salmonella* (179).

In contrast to other species, no genome-wide expression profiles in the chicken in response to *Salmonella* have been measured. Therefore several processes in the chicken host in response to *Salmonella* may be unidentified. Furthermore validation of the *in vitro* gene expression observations by *in vivo* data are scarce. In addition it is not known why some chicken lines are more susceptible to *Salmonella* infections than others (13, 62).In the present study we describe the gene expression response in the intestine of young chickens after a *Salmonella* infection. The data obtained with a whole genome oligonucleotide array were compared to those obtained with a tissue specific cDNA array. Also two different chicken lines were used and the results were compared with each other to determine the role of the genetic background in the host response.

Materials and methods

Array Fabrication

The oligonucleotide arrays were obtained from Affymetrix, the GeneChip Chicken Genome Array. These arrays contained 38,449 *Gallus gallus* probe sets with 11 probe pairs of 25-mers per sequence.

The cDNA microarrays were constructed as described earlier (183). The latter microarrays contained 3072 cDNAs from a subtracted and normalized intestinal library and 1152 cDNAs from a subtracted and normalized concanavalin A stimulated spleen library. All cDNAs were spotted in triplicate on each cDNA microarray.

Chicken lines

The following study was approved by the institutional Animal Experiment Commission in accordance with the Dutch regulations on animal experimentation. Two meat type chicken lines (*Gallus gallus*), the fast growing line A and the slow growing line B were used in the present study (Nutreco®). These chicken lines differ in response to *Salmonella*. Line A had a more severe systemic infection, as after a *Salmonella* infection more CFU in the liver were found and the clearance was slower than for line B. In addition the chickens from line A had a higher weight gain depression after a *Salmonella* infection compared to the chickens from line B (186).

Experimental infection

Ten one-day old chickens of each line (A and B) were randomly divided into 2 groups. After hatching, it was determined that birds were free of *Salmonella*. Of each chicken line, five chickens of 1-day old were orally inoculated with 0.2 ml of a bacterial suspension containing 10^5 CFU *S.* serovar Enteritidis phage type 4 (nalidixic acid resistant). Five chickens of the control group were inoculated with 0.2 ml saline. The five chickens of each group were sacrificed 24 h post infection. For detection of *S.* serovar Enteritidis a cloacal swab was taken and after overnight enrichment it was spread on brilliant green agar + 100 ppm nalidixic acid for *Salmonella* determination (37°C, 18-24 h). One gr of liver of each bird was homogenized in 9 ml Buffered Peptone Water (BPW), serial diluted in BPW, and plated onto brilliant green agar with nalidixic acid for quantitative *S.* serovar Enteritidis determination (37°C, 18-24 h) by counting the colony forming units. Pieces of the jejunum were snap frozen in liquid nitrogen and stored at -70°C.

RNA isolation

Pieces of the jejunum were crushed under liquid nitrogen. 50-100 mg tissues of the different chicks were used to isolate total RNA using TRIzol reagent (Invitrogen), according to instructions of the manufacturer with an additional step. The homogenized tissue samples were resuspended in 1 ml of TRIzol Reagent using a syringe and 21 gauge needle and passing the lysate through 10 times. After homogenisation, insoluble material was removed from the homogenate by centrifugation at 12,000 × g for 10 min at 4°C. For the array hybridisation pools of RNA were made in which equal amounts of RNA from five different chickens of the same line and condition were present. For the oligonucleotide arrays an extra purification was performed with NucloSpin RNA II (Macherey-Nagel).

Hybridising, Scanning and Analysis of the Oligonucleotide Microarray

The Affymetrix One Cycle Target Labelling Kit was used to synthesize the biotin-cRNA. Labelling of 20 µg of the RNA, hybridization, staining, washing steps, and array scanning were carried out following Affymetrix protocols. Labelled cRNA was checked using the Agilent bioanalyzer.

Hybridising, Scanning and Analysis of the cDNA Microarray.

Before hybridisation, the cDNA microarray was pre-hybridised in 5% SSC, 0.1% SDS and 1% BSA at 42°C for 30 min. To label the RNA, the MICROMAX TSA

labelling and detection kit (PerkinElmer) was used. The TSA probe labelling and array hybridisation were performed as described in the instruction manual with minor modifications. Biotin- and fluorescein-labelled cDNAs were generated from 5 µg of total RNA from the chicken jejunum pools per reaction. The cDNA synthesis time was increased to 3 h at 42°C, as suggested (84). Post-hybridisation washes were performed according to the manufacturer's recommendations. Hybridisations were performed in duplicate with the fluorophores reversed. After signal amplification, the microarrays were dried and scanned for Cy5 and Cy3 fluorescence in a Packard Bioscience BioChip Technologies apparatus. The image was processed with Genepix pro 5.0 (Genomic Solutions) and spots were located and integrated with the spotting file of the robot used for spotting. For each cDNA six values were obtained, three for one slide and three for the dye-swap. Genes with two or more missing values were removed from further analysis. Missing values were possibly due to a bad signal to noise ratio. A gene was considered to be differentially expressed when the mean value of the ratio was > 1.00 or < -1.00. Because the ratio on a cDNA array is expressed in a log2 scale, a ratio of > 1.00 or < -1.00 corresponds to a more than twofold up- or downregulation respectively. To compare with the oligonucleotide array and the quantitative PCR, the $\log_2$ ratios were transformed to normal ratios. Bacterial clones containing an insert representing a differentially expressed gene were sequenced and analysed using Seqman as described (184).

All microarray information has been submitted into the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) website (http://www.ncbi.nlm.nih.gov/geo/). The experiment series has been submitted as GSE3702.

Statistics

The oligonucleotide microarray was analysed with the Rosetta Luminator software package. Genes identified with a p value < 0.01 were considered statistically significant. Genes on the cDNA microarray were identified with significance analysis of microarrays (based on SAM (180)) with a false discovery rate < 1%.

Results

Clinical symptoms of the Salmonella infection

24 h after oral inoculation the chicks were sacrificed. The cloacal swaps of the control animals were free of *Salmonella*, while the cloacal swaps of all *Salmonella* inoculated chicks were positive. Furthermore, the *Salmonella* infected birds from both

lines showed already signs of systemic disease as *Salmonella* was detectable in the liver. There were no differences in weight between the different groups.

Regulated genes in both chicken lines in response to the Salmonella infection

To investigate a general response in the chicken intestine to the *Salmonella* infection, we concentrated on more than twofold differences in gene expression 24 h after the *Salmonella* infection in both chicken lines. Genes were identified to be regulated in response to the *Salmonella* infection using the Affymetrix GeneChip array and a p value <0.01. In line A in total 237 genes had more than twofold expression differences when control chicken were compared to infected birds, 183 genes were upregulated and 54 were downregulated. In Line B, 115 genes were regulated, 91 induced and 24 repressed. Of the induced genes, 13 were found in both chicken lines (Table 1), of the repressed genes no similar regulation of genes in both lines was found.

The same criteria of gene analysis were used with the data of the cDNA microarray, at least twofold up- or downregulation and a p value <0.01. In line A 39 induced and 23 repressed genes were identified, in line B 45 induced and 45 repressed genes were identified. Five corresponding genes were upregulated in both chicken lines (Table 1), no corresponding downregulated genes were found in the two lines. The sequences of the five genes identified with the cDNA array were compared with the sequences of the GeneChip array to compare the two platforms. For all five genes identified with the cDNA array, sequences were present on the GeneChip array (Table 2). One of these genes, lysozyme G, was also already identified to be upregulated more than twofold in both lines with the GeneChip array. Three of the five genes found with the cDNA array, cytidine deaminase, similar to DNA segment, Chr 10 ,and similar to fatty acid synthase were also significantly ($p<0.01$) upregulated as analysed with the GeneChip array, but less than twofold. Dickkopf homolog 3 identified as upregulated gene with the cDNA array was not found to be differently regulated in the two chicken lines after the *Salmonella* infection with the GeneChip array.

To further compare the results obtained with both platforms, the expression data of 71 randomly chosen sequences of the cDNA microarray were compared with the results of the oligonucleotide microarray. For line A 36/71 sequences gave similar results between the two platforms e.g. upregulation, downregulation or no regulation. For line B 25/71 sequences gave similar results.

Table 1. Genes more than two-fold regulated in both chicken lines in response to *Salmonella* infection, with p<0.01 for the microarray data.

Accession no.	Locus ID*	Description	Fold-change	
GeneChip array			Line A	Line B
NM_205320	396260	mature avidin	64.3	3.9
CF250837	418700	similar to lysozyme (EC 3.2.1.17) g	8.4	19.2
	418700	similar to lysozyme (EC 3.2.1.17) g	6.2	12.8
X61198	395708	lysozyme	7.2	4.7
BU435658	HLA-DRB1	MHC class II beta chain (B-LB) mRNA, B-LB-B21 allele	2.1	55.9
BU260479	423432	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10	7.8	10.6
NM_204989	395837	fibrinogen gamma chain (FGG), mRNA	6.9	100
BX932101	396241	ovotransferrin	2.2	15.1
BU220239	416546	similar to NADPH oxidase organizer 1 isoform b; regulatory protein P41NOX; Nox organizer 1	18.6	3.7
AJ721110	421702	similar to Vascular non-inflammatory molecule 3 precursor (Vanin 3)	10.5	3.1
NM_205213	396135	hepatocyte growth factor-like/macrophage stimulating protein (HGF1/MSP), mRNA	8.9	3.3
BM425681	417345	hypothetical gene supported by CR391572	6.3	2.8
XM_418660	420559	similar to KIAA2005 protein	2.0	2.5
cDNA array				
NM_204933	395773	cytidine deaminase (CDD), mRNA	2.4	2.4
NM_205125	396023	dickkopf homolog 3	5.3	6.8
XM_420282	422305	similar to DNA segment, Chr 10, Johns Hopkins University 81 expressed	6.1	7.1
XM_416896	418700	similar to lysozyme (EC 3.2.1.17) g	2.3	6.2
XM_418586	420484	similar to fatty acid synthase	2.3	2.4

* The locus ID refers to the LocusLink and Entrez Gene databases from the NCBI.

Table 2. Comparison of the cDNA microarray with the Genechip array for five genes. Values are expression levels *Salmonella* infected animals/ control animals.

Gene Name	GeneChip identifier	line A		line B	
		cDNA	GeneChip	cDNA	GeneChip
cytidine deaminase	Gga.458.1.S1_at	2.4	1.3*	2.4	1.2*
	Gga.458.1.S1_s_at		1.5*		1.2*
dickkopf homolog 3	Gga.3573.2.S1_a_at	5.3	1.0	6.8	1.0
similar to DNA segment, Chr 10, Johns Hopkins University 81 expressed	GgaAffx.20699.2.S1_s_at	6.1	7.0*	7.1	1.6*
similar to lysozyme (EC 3.2.1.17) g	Gga.9103.1.S1_at	2.3	8.4*	6.2	19.2*
	GgaAffx.25059.1.S1_at		6.2*		12.8*
similar to fatty acid synthase	GgaAffx.4651.1.S1_at	2.3	6.9*	2.4	1.7*
	GgaAffx.4657.1.S1_s_at		3.7*		1.8*

* Ratios found with GeneChip microarray with $p < 0.01$. All genes in this list had a $p < 0.01$ for the ratios found with the cDNA microarray.

Highly regulated genes

With the primary focus on regulation in both chicken lines, the acquired gene lists did not include a number of highly regulated genes in only one of the chicken lines. Therefore in the next stage, we concentrated our analyses to genes up- or downregulated more than ten fold in only one chicken line 24 h post infection with *Salmonella*. With the GeneChip array in line A 13 highly upregulated genes were identified, in line B 32 genes were more than 10 fold upregulated (Table 3). No ten fold downregulation of genes was found, nor were more than 10 fold differences in expression identified with the cDNA microarray. The more than 10 fold upregulated genes after the *Salmonella* infection identified with the GeneChip could be grouped into different processes. In line A, the upregulated genes encoded for proteins involved in inflammation or had an unknown function. Upregulated genes in line B genes encoded for proteins involved in the fibrinogen system, genes encoding acute phase proteins, genes involved in actin polymerisation, in cell-cell interaction or genes with an unknown function.

Table 3. Genes more than 10-fold upregulated in one of the chicken lines determined with the GeneChip arrays.

Accession no.	Locus*	Description	line A	line B
inflammation				
XM_420284	422307	similar to putative XIRG protein	17	n.s.
NM_204259	374110	prostaglandin D2 synthase 21kDa (brain) (PTGDS)	17.9	n.s.
XM_422310	424467	similar to vascular cell adhesion molecule (VCAM)	14.3	n.s.
XM_425699	428141	similar to Trappin-6	14	n.s.
X61200		126 MRP	15.7	n.s.
fibrinogen system				
NM_205356	396307	pre-fibrinogen alpha subunit	n.s.	30.5
NM_205356	396307	pre-fibrinogen alpha subunit	n.s.	16.8
XM_420369	373926	gallus gallus fibrinogen, B beta polypeptide	n.s.	25.4
XM_419618	421580	similar to Plasminogen	n.s.	21.5
S79838	396077	antithrombin	n.s.	10.8
XM_426150	428593	similar to plasmin (EC 3.4.21.7) precursor - rhesus macaque	13.3	n.s.
acute phase protein				
AY534895	414342	gallinacin 7 prepropeptide	n.s.	47
NM_207180	395364	PIT 54 protein	n.s.	43.5
NM_207180	395364	PIT 54 protein	n.s.	32.8
AY584568	395220	alpha 1-acid glycoprotein (ogchi)	n.s.	14.7
NM_205405	396370	complement C3 alpha chain	n.s.	14.5
XM_421343	423434	similar to alpha-1-antitrypsin	n.s.	23.4
XM_421343	423434	similar to alpha-1-antitrypsin	n.s.	13
XM_414253	415901	similar to inter-alpha-trypsin inhibitor heavy chain3	n.s.	12.7
NM_205261	396197	preproalbumin (serum albumin)	n.s.	91
XM_422764	424956	similar to fetuin	n.s.	57.4
NM_205168	396080	secreted phosphoprotein 24	n.s.	43.1
XM_415683	417431	similar to apolipoprotein H precursor	n.s.	40
NM_205238	396166	retinol-binding protein 4, plasma	n.s.	28.9

Table 3 (continued)

NM_205304	396241	ovotransferrin	n.s.	14.7
NM_205335	396277	transthyretin	n.s.	12.9
actin cytoskeleton				
L02622	396417	intestinal zipper protein	n.s.	11.4
NM_213577	404755	thyroid hormone responsive spot 14 alpha	n.s.	42.3
unknown				
NM_205422	396393	quiescence-specific protein (P20K)	100	n.s.
XM_421011	423079	similar to Hermansky-Pudlak syndrome 5 protein (Alpha-integrin-binding protein 63) (Ruby-eye protein 2 homolog) (Ru2)	33.2	n.s.
XM_420627	422672	similar to VLLH2748	15	n.s.
XM_420283	422306	similar to cDNA sequence BC023823	11.9	1.7
XM_414491	416159	similar to Potential phospholipid-transporting ATPase VB	11.1	n.s.
CK613373		cDNA clone LPSk_F01	11	n.s.
DQ018754		clone AY002 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	11	n.s.
X56659	396292	Chicken Col6A2 gene for type VI collagen subunit alpha2	n.s.	47.2
BX932086		finished cDNA, clone ChEST158l24	n.s.	39.3
NM_001006264	418639	phosphoribosyl pyrophosphate synthetase 2	n.s.	34.9
NM_204634	395345	liver basic fatty acid binding protein	n.s.	22.2
XM_415786	417540	similar to phosphoserine phosphatase; L-3-phosphoserine phosphatase; O-phosphoserine phosphohydrolase; PSPase	n.s.	15.7
BX935051		finished cDNA, clone ChEST70a22	n.s.	13.3
XM_430095	423766	hypothetical gene supported by CR390034	n.s.	12.1
XM_415144	416852	similar to 4-hydroxyphenylpyruvate dioxygenase	n.s.	11.7
NM_001006264	418639	phosphoribosyl pyrophosphate synthetase 2	n.s.	10.7
CR387055		finished cDNA, clone ChEST124l16	n.s.	10.3

* The locus ID refers to the LocusLink and Entrez Gene databases from the NCBI.
n.s. no statistical differences in expression levels.

Cytokines

No cytokines were identified with the cDNA microarray nor with the GeneChip microarray in the previous analysis. To evaluate the gene expression differences of immune-related genes, 7 cytokines and chemokines were investigated using the GeneChip array. The results are shown in Table 4. Five of these genes, IFN-γ, IL-12, IL-13, IL-4 and IL-6 were not significantly regulated in either chicken line. However, IL-8 and K60 were upregulated in line A, but not in line B.

Table 4. Gene expression differences found for different cytokines in response to the *Salmonella* infection in two chicken lines.

gene	affymetrix Code	Accession no.	line A		line B
IFN-gamma	Gga.916.1.S2_at	NM_205149	-2.3		-1.2
	Gga.916.1.S1_at	AY163160	-3.8		-2.3
IL12B	Gga.14433.1.S1_at	NM_213571	-1.8		2.1
	Gga.14433.1.S2_at	AJ564201	1.2		-2.5
IL13	GgaAffx.4230.1.S1_at	ENSGALG00000006801.1	1.5		-2.0
IL4	GgaAffx.22064.1.S1_s_at	ENSGALG00000006827.1	1.2		1.1
	GgaAffx.22064.1.S1_at	ENSGALG00000006827.1	-1.0		-2.0
	Gga.5637.1.S1_at	BU459082	-2.0		1.6
IL6	Gga.2769.1.S1_at	NM_204628	2.9		-1.2
	Gga.6815.1.A1_at	BX273308	-1.8		-1.6
IL8	Gga.826.1.S1_s_at	M16199	6.2	p<0.01	1.3
K60	Gga.512.1.S1_at	NM_205018	3.5	p<0.01	1.4

Discussion

So far few articles are published studying gene expression profiles in the chicken and each study used a different array platform. This makes it difficult to interpret and compare results from different experiments. Recently the first draft genome sequence of the red jungle fowl, *Gallus gallus*, has become available (74). Earlier a large amount of EST data was already available (1). These data were used to construct a whole genome GeneChip Chicken Genome Array. This array contains comprehensive coverage of 32773 transcripts corresponding to 29268 chicken and chicken viral pathogen genes. A disadvantage of this array is that it is based on the first temporary

data, missing newly identified genes. Unfortunately, a number of immune-related genes are recently shown not to be recognized in the initial annotation or were wrongly annotated (80), therefore they are not present on the array.

The cDNA microarray was intestine and T-cell specific and made from subtracted and normalised cDNA libraries. A disadvantage of the used cDNA array is that not all cDNAs sequences are known, only the ESTs that were interesting due to their expression profile in this or earlier experiments are sequenced (184, 186). Therefore it is not known whether a gene is not present on the array or was not analysed before. However, genes identified with the cDNA array could be compared with the GeneChip array. The results obtained with the two platforms were moderately comparable, with about half of the genes giving similar results for both platforms. Almost half of the genes with different results between the two platforms had low expression levels. For these genes up- or downregulation could be detected with the cDNA microarray, but not with the GeneChip array. So the cDNA microarray could detect differences in expression at lower expression levels compared to the GeneChip array. For the genes upregulated in both chicken lines after the *Salmonella* infection, the results of the two platforms where more comparable, with expression differences confirmed for 4 out of the 5 genes. It has been shown before that different microarray platforms does not always give the same results (148, 201).

The intestine is a complex system to study *in vivo*, due to the different cell types and multiple functions. Upon encounter with a pathogen many processes take place simultaneously in the intestine like different stages of immune responses, wound healing, influx of heterophils and inflammation processes To highlight the responses after a Salmonella infection in cellular context the Gene expression in the whole intestine of 1-day old chickens was investigated with the micro-array technology as is reviewed (7). The advantage of investigating the gene response *in vivo* above *in vitro* infection in cell lines is that the cellular context is remained. This context is important to be able to identify complex cellular regulation. As expected genes were identified to be upregulated in response to the *Salmonella* infection in both chicken lines. Some of these genes encode proteins involved in the innate immune system, like lysozyme and similar to lysozyme G and the acute phase proteins avidin, antitrypsin, fibrinogen and ovotransferrin. Avidin is shown before to be upregulated after intestinal injury in chicken, but not in rat and mice (49). Other upregulated genes after *Salmonella* encode for NOXO1, MHC class II, Vanin 3, HGF1/MSP and several with unknown homology or function. NOXO1 is involved in innate host defense processes as it activates Nox1, which produces superoxide, a precursor of reactive oxygen species (56) . MHC class II

is also upregulated in both lines, a molecule which among others is expressed on macrophages that are important in the innate immune system. Vanin 3 has possibly a role in processes pertaining to tissue repair in the context of oxidative stress (110). HGF1/MSP has a role in wound healing (156). Overall, gene expression profiles indicate that the innate immune system and tissue repair is activated in the intestine of both chicken lines in response to the *Salmonella* infection. This is the host response that is expected after infection with a bacteria (60).

Beside the acute phase proteins upregulated in both chicken lines, there was a large set of acute phase proteins highly upregulated in line B only. Acute phase proteins exert a large array of adaptive effects on the inflammatory process (including minimizing the extent of local tissue damage), immune cell function, and tissue repair. Although many of the acute phase proteins predominantly originate from the liver, it is also shown that many of the acute phase plasma proteins are expressed in human intestinal epithelial cell lines Caco2 and T84. Possibly, enterocytes are involved in a local response in injury/inflammation at the epithelial surface (121). It is quite surprising that all the acute phase mRNAs are upregulated. It is known that serum levels of some of these proteins rise after inflammation, like fibrinogen, alpha1-antitrypsin, complement C3, but serum levels of other proteins decrease, like albumin and transferrin. However, serum levels of proteins in the blood does not necessarily corresponds to mRNA levels in the gut. Furthermore, there is evidence that chicken transferrin levels in serum increase in response to inflammation (202), whereas in mammals the level of transferrin decreases; therefore it is possible that chickens reacts differently than mammals to inflammation on the level of acute phase proteins.

Another large set of upregulated genes in line B is related to the fibrinogen system. Fibrinogen itself is also an acute phase protein (194). Fibrinogen is mainly synthesized in the liver, but it is also synthesized at several extrahepatic sites as intestinal epithelial cells secrete small amounts of fibrinogen (194). Fibrinogen is cleaved by thrombin and converted to insoluble fibrin polymer, which functions in wound healing. The upregulation of antithrombin found in chicken line B can reduce intestinal injury, by both its anticoagulant and anti-inflammatory effects (162). Antithrombin causes a decrease in free thrombin, thereby reducing the formation of fibrin protein. The fibrin polymer can be degraded by the action of plasmin. The levels of precursors of plasmin increased, also reducing the formation of fibrin protein. However, the levels of the precursor of fibrin, fibrinogen increase, as the alpha, beta and gamma chains of fibrinogen increase. Probably in the intestine these molecules have other functions besides the formation of fibrin. It was remarkable that in chicken

line A also a plasmin precursor was upregulated, but not the same as in line B. Both genes are located directly next to each other in the same orientation on chromosome 3, but apparently are differently regulated.

In line B, two upregulated genes were identified that were involved in actin cytoskeleton. It is known that *Salmonella* strains have virulence mechanisms to subvert the actin cytoskeleton and promote infection (63). For intestinal zipper protein a role in regulating the interaction of brush border myosin 1 with the actin core of the microvillus was suggested (22) whereas thyroid hormone responsive spot 14 alpha is homologue to mouse Mid1ip1, which is negatively regulated microtubule depolymerization (20). Actin polymerisation is not a surprising cellular event to be altered after a *Salmonella* infection, as *Salmonella* has evolved sophisticated mechanisms to subvert the cytoskeletal machinery of its host (135). Possibly, the upregulation of intestinal zipper protein and thyroid hormone responsive spot 14 alpha influences the invasion or transmigration of *Salmonella*. Transmigration of *Salmonella* will possible be a different process of timing in both lines.

In line A, a number of upregulated genes were identified that were not regulated in line B. From some of these genes it is known that their expression increases during inflammation processes. For instance, expression of mouse IRG1 induced in cultured macrophages is a response to bacterial lipopolysaccharide treatment (161). Prostaglandins play a role in *Salmonella* induced enteropathogenic responses (191). VCAM is expressed in the submucosa of intestinal tissues exposed to an inflammatory stimulus (193). Trappins are thought to play an important role in the regulation of inflammation and protection against tissue damage (159). This could indicate that translocation and intestinal lesions are differently regulated at day 1 after infection between both chicken lines. The human homologue of 126 MRP, MRP14 is abundantly expressed in neutrophils and the levels of this protein are elevated in body fluids of inflamed tissues (126). Heterophils, the chicken homologue of neuterophils, are known to differ in activity between chicken lines that differ in *Salmonella* susceptibility (177), which might indicate that between both used chicken lines the heterophil defense mechanisme differ. All these findings together suggest that in line A, but not in line B, known inflammatory processes are regulated in the intestine 24 hours after the *in vivo* *Salmonella* inoculation.

In other *in vivo* studies cytokines were described as important in the early host response to *Salmonella*. However, although a number of genes involved in inflammation and innate immunity were upregulated in our study, no cytokines and

chemokines were identified in our screen. Therefore, we concentrated on cytokines described earlier. Of the 7 genes examined, we found upregulation of the cytokine IL-8 and the CXC chemokine K60 in the jejunum of line A in response to the *Salmonella* infection. This suggests a proinflammatory regulation in the intestine of line A. Interestingly upregulation of IL-8 and K60 was also found in another study (196). Both studies inoculated 1 day old chickens with *Salmonella* and studied early responses; however, we used *Salmonella* enterica serovar Enteritidis whereas in the other study serovar Typhimurium was used. Other cytokines described in other studies showed no marked differences in expression in this study, which could be due to other timepoints of expression, other tissues or the use of older or genetically different chickens.

Strikingly, more differences than similarities were found in the gene expression profiles of the two chicken lines in response to the *Salmonella* infection. Our two different broiler lines had different growth rates, and it was suggested that they respond differently to a *Salmonella* infection (62). Indeed line-A responded differently to a *Salmonella* infection compared to line-B determined by the difference in liver clearance and weight gain depression over time (186).

This study has identified new genes involved in the chicken host response to a *Salmonella* infection and revealed some of the complex *in vivo* interactions in the intestine after encounter with a pathogen. Furthermore, it has shown that genetic background is of major importance for early gene expression responses as significant differences between chicken lines in the response to a *Salmonella* infection has been found. Important processes after an *in vivo Salmonella* infection based on gene expression responses are: inflammation, wound healing, acute phase response, the fibrinogen system, and actin polymerisation. Further studies will be needed to answer the question whether the upregulated genes in the chicken intestine are general upregulated to an infection, or are *Salmonella* specific. For some genes, like quiescence-specific protein, MHC class II and ovotransferrin it is already shown that their expression is induced in chicken embryo fibroblast as a response to Marek's Disease Virus (124), suggesting that these proteins in chicken are generally induced by pathogenic microorganisms. Other genes might be more specific for certain types of bacteria. In addition there remains a series of unknown genes for which their function in host response on a infection needs to be determined further.

Chapter 6.

Immunological and gene expression responses to a *Salmonella* infection in the chicken intestine

Saskia van Hemert
Arjan Hoekman
Mari Smits
Annemarie Rebel

Veterinary Research (2007) 38: 51-63

Abstract

Besides infection in humans, *Salmonella enteritidis* can also cause serious illness in young chickens. However, the genetic and immunological parameters important for the disease in chickens are not well characterized. In this study processes in the chicken intestine in response to a *Salmonella* infection were investigated in two different chicken lines. One-day-old chickens were orally infected with *Salmonella* T-cell subpopulations, phagocytic properties of intestinal mononuclear cells and RNA expression levels of the jejunum were investigated. The two chicken lines differed in the amount of cfu in the liver and the growth retardation after the infection. Differences in phagocytic activity of intestinal mononuclear cells were found between control and *Salmonella* infected chickens. The number of $CD4^+$ T-cells of the intestine decreased after the *Salmonella* infection in one chicken line, while the number of $CD8^+$ T-cells increased in both chicken lines, but the time p.i. of this increase differed between the lines. In one chicken line the expression levels of the genes carboxypeptidase M and similar to ORF2 decreased after the *Salmonella* infection, which might be related to a decrease in the amount of macrophages. With the microarray ten genes were found that were regulated in only one of the chicken lines, while we found six genes regulated in response to the infection in both chicken lines. So differences in genetic background of the chickens influences the intestinal host response of the *Salmonella* infection as observed by phagocytic activity, gene expression and changes in the number of T-cell subpopulations and macrophages.

Introduction

Salmonella enterica is one of the most common causes of food poisoning in humans, mostly caused by poultry products infected by *S. enterica* serovars Typhimurium or Enteritidis (137). In addition to the enteric disease in humans *Salmonella* serovars Typhimurium and Enteritidis are also capable of causing severe systemic disease in newly hatched chicks and in birds under extreme stress conditions. (176). In young chickens infection with *Salmonella* leads to diarrhea and intestinal lesions and to an influx of heterophils into the gut accompanied by inflammation and damage to villi (13).

An infection with *Salmonella* usually starts by ingestion, followed by colonization in the intestine. After colonization, *Salmonella* is able to penetrate the mucosal epithelium which results in a systemic infection, with colonization of the spleen and liver (69). The immunological responses in the chicken intestine to a *Salmonella* infection are not fully understood. In general, the innate immune system plays an important role in the early response to *Salmonella*. Upon oral challenge with *Salmonella* the area occupied by the macrophages in the caecal wall was increased in neonatal chickens (187). Macrophage-derived cytokines and immune mediators can initiate local and systemic inflammatory responses. This local inflammation likely explains the strong influx and continued presence of macrophages and T-cells after a *Salmonella* infection (187). It was also shown that after infection with *Salmonella* the number of $CD8^+TCR\gamma\delta^+$ T-cells in the chicken intestine increased (19). $CD4^+$ or $CD8^+$ T-cell responses are not required for the early host response (112), but $CD4^+$ T-cell responses, particularly Th1 responses, play an important role in the clearance of *Salmonella* from the gastrointestinal tract (16, 73, 199).

Earlier gene expression studies suggested that innate immunity, inflammation and T-cell responses are important processes in the chicken intestine in response to a *Salmonella* infection (185). However, these findings were only based on gene expression data of one chicken experiment. To further evaluate immune responses in the one-day-old chicken intestine after a *Salmonella* infection, differences in T-cell populations were investigated as were phagocytic properties of intestinal mononuclear cells and RNA expression.

Material and Methods

Chickens

Two meat type chicken lines, a fast growing line F and a slow growing line S were used in the present study (Nutreco®, Boxmeer, The Netherlands). Line S is a commercial dam line from white plymouth rock origin. Line F is an experimental line selected for egg production, liveability and slow growth to be used in future for processing of broilers of 80 days and older. As indicated in the results section, these lines differed in growth retardation and amount of colony forming units in the liver after *Salmonella* infection. 80 one-day old chickens of each line (F and S) were randomly divided into 2 groups, 40 chickens each. After hatching, birds were checked to be free of *Salmonella*.

Experimental infection

Salmonella enterica serovar Enteritidis phage type 4 (nalidixic acid resistant) was grown in buffered peptone water (BPW) overnight while shaking. Of each chicken line, one group of 1-day old chickens was orally inoculated with 0.2 ml of the bacterial suspension containing 10^5 cfu *S. enterica* serovar Enteritidis. The control groups were inoculated with 0.2 ml saline. Ten chickens of each group were randomly chosen, weighed and sacrificed at day 1, 5 and 7 post infection (pi). Pieces of the jejunum were snap frozen in liquid nitrogen and stored at -70°C for immunohistochemistry and RNA isolation. The remaining part of the jejunum was stored in buffered saline at 4°C until isolation of intestinal mononuclear cells. The liver was removed and weighted and kept at 4°C until bacteriological examination. At day 9 the chickens were weighted only and at day 12 the chickens were weighted and bacteriological examinations were performed, but no mononuclear cells or RNA was isolated. The study was approved by the institutional Animal Experiment Commission in accordance with the Dutch regulations on animal experimentation.

Bacteriological examination

For detection of *S.* serovar Enteritidis a cloacal swab was taken and after overnight enrichment it was spread on brilliant green agar + 100 ppm nalidixic acid for *Salmonella* determination (37°C, 18-24 hr). One gram of liver of each bird was homogenized in 9 ml Buffered Peptone Water (BPW), serial diluted in BPW, and plated onto brilliant green agar with nalidixic acid for quantitative S. serovar Enteritidis determination (37°C, 18-24 hr) by counting the colony forming units. To identify

significant differences between the two chicken lines a student-t test was performed on the log-transformed data.

Intestinal mononuclear cells isolation

The jejunum was opened longitudinally, washed with phosphate buffered saline (PBS) and cut into pieces of 1 cm. These pieces were incubated at 37° for 45 minutes in Medium I (PBS containing 1mM EDTA and 5mM DTT). The suspension contained the intraepithelial cells (fraction 1) and was kept at 4°C until use. The remaining pieces of intestine were further incubated at 37°C for 90 minutes in Medium II (RPMI + 5% fetal calf serum + 400 FALGPA units Collagenase per liter (Sigma, St Louis, MO, USA) + 60000 Kunitz units DNase I per liter (Sigma, St Louis, MO, USA)) while shaking (fraction 2, lamina propria cells). The two fractions were mixed and after centrifugation for 10 minutes at 460 × g the pellet was resuspended in 10 ml Medium III (RPMI + 1% Fetal calf serum +60000 Kunitz units DNase I per liter). The suspension was purified on a 25% percoll (Sigma, St Louis, MO,USA) gradient centrifuging for 15 minutes at 2000 rpm. The pellet was washed twice with PBS and cells were coloured with 0.1% trypan blue and viable (unstained) cells were counted. The cells were resuspended in PBS at a concentration of 1 x 10^6 cells per ml.

Phagocyte activity of intestinal mononuclear cells

The intestinal mononuclear cell isolates were tested for their phagocytic activity by intake of live *Salmonella enteritidis* phage type 4 as described by Kramer *et al*. (91). Briefly, the gut mononuclear cell suspension was diluted to 1×10^7 cells/ml in RPMI. 1 ml of *Salmonella enteritidis* (overnight culture 1:100 diluted and grown for approximately 3 hours, about 1×10^8 cfu) was added and the mixture was incubated for 45 min at 37°C. Subsequently, 200 μg gentamycin was added to kill non-internalised bacteria and incubated for 45 min at room temperature. After washing in PBS the cells were lysed by adding 1 ml 0.2% saponine in PBS and incubating 5 minutes to release the bacteria internalised by the phagocytic intestinal mononuclear lymphocytes. The number of S. enteritidis internalised by the cells was counted on BGA-NaI^+ plates. A higher value indicated a higher phagocytic activity of the mononuclear cells.

Flow cytometry

The total leukocyte subpopulation of the intestinal mononuclear cell isolates was estimated by flow cytometry. For the flow cytometric analysis the concentration of the isolated cells was brought to 20 x 10^6 cells/ml, and 50 μl was transferred into a 96 well

plate on ice. Cells were washed with PBS supplemented with 1% Foetal Calf Serum (FCS). A normal mouse serum (1%) was applied to block non-specific binding sites, followed by adding the monoclonal antibody CD45-PE (Southern Biotech, Birmingham, Alabama, USA). After 15 min incubation at 4°C, the cells were washed twice with PBS/FCS and re-suspended in 200 µl ice-cold PBS/FCS. A total of 10^4 cells per sample were analysed by flow cytometry (FACS Calibur™)(Beckton Dickinson, Leiden, The Netherlands). The data were analysed using a flow cytometry computer programme.

Immunohistochemistry

Frozen jejunum section collected at day 1, 5 and 7 pi were stained for $CD4^+$ T-cells, $CD8^+$ T-cells and macrophages. Immunohistological staining by an indirect immunoperoxidase method was performed on frozen tissue sections (10 μm thick). The sections were loaded on glass slides, air-dried, and fixed in acetone for 10 min. After being dried, the slides were immersed in PBS with 0.1% BSA and were subsequently incubated for 1 h with monoclonal antibodies against macrophages (1:500 CVIChNL68.1(78)), $CD4^+$ T cells (1:200 CT-4 Southern Biotech), or $CD8^+$ T cells (1:200 CT-8 Southern Biotech) followed by peroxidase-conjugated rabbit anti-mouse Ig (1:80 Dakopatts, Denmark). Peroxidase activity was detected by 0.05% 3,3-diaminobenzidine (DAB) in 0.1 M Tris-HCl solution (pH 7.5) containing 0.03% H_2O_2. The coupes were further coloured with 1% $CoCl_2$ for 5 minutes. After washing the nuclei were counter-stained with hematoxylin. The sections were dehydrated and mounted in distyrene-tricresyl phosphate-xylene (DPX). The images were acquired and analysed with Image-Pro Plus (version 5.1, media cybernetics).

RNA isolation

Pieces of the jejunum were crushed under liquid nitrogen. 50-100 mg tissues of the different chicks were used to isolate total RNA using TRIzol reagent (Invitrogen, Breda, the Netherlands), according to instructions of the manufacturer with an additional step. The homogenized tissue samples were resuspended in 1 ml of TRIzol Reagent using a syringe and 21 gauge needle and passing the lysate through 10 times. After homogenisation, insoluble material was removed from the homogenate by centrifugation at 12,000 × g for 10 minutes at 4°C. For the array hybridisation pools of RNA were made in which equal amounts of RNA from ten different chickens of the same line, condition and timepoint were present.

Hybridising of the microarray

The microarrays were constructed as described earlier (183). The microarrays contained 3072 cDNAs from a subtracted intestinal library and 1152 cDNAs from a concanavalin A stimulated spleen library. All cDNAs were spotted in triplicate on each microarray. Before hybridisation, the microarray was pre-hybridised in 5% SSC, 0.1% SDS and 1% BSA at 42°C for 30 minutes. To label the RNA, the MICROMAX TSA labelling and detection kit (PerkinElmer, Wellesley, MA) was used. The TSA probe labelling and array hybridisation were performed as described in the instruction manual with minor modifications. Biotin- and fluorescein-labelled cDNAs were generated from 5 µg of total RNA from the chicken jejunum pools per reaction. The cDNA synthesis time was increased to 3 hours at 42°C. The generated cDNA was transferred to a microcon YM-100 centrifugal filter cartidge (Millipore, Billerica, MA) and washed twice with 10 mM Tris-HCl, pH 7.5. The cDNA was resolved in 60 µl hybridisation buffer from the kit and heated to 95°C for two minutes. Hybridisation of the array was done during 16-20 hours at 65°C. Post-hybridisation washes were performed according to the manufacturer's recommendations. Hybridisations were performed in duplicate with the fluorophores reversed. After signal amplification the microarrays were dried and scanned for Cy5 and Cy3 fluorescence in a Packard Bioscience BioChip Technologies apparatus. The image was processed with Genepix pro 5.0 (Genomic Solutions, Ann Arbor, MI) and spots were located and integrated with the spotting file of the robot used for spotting. Reports were created of total spot information and spot intensity ratio for subsequent data analyses.

Analysis of the microarray data

Each spot was corrected for local background and the data for each slide were normalized so that the mean of the ratio of all spots was equal to one with the GenePix Pro 5.0 program. A total of 8 microarrays were used in this experiment. The following four comparisons were made using pools of RNA from ten different chickens:1- line F (fast growing) control vs. line S (slow growing) control, 2- line F *Salmonella* vs. line S *Salmonella*, 3- line F control vs. line F *Salmonella*, and 4- line S control vs. line S *Salmonella*. For each comparison six values were obtained per gene, three for one slide and three for the dye-swap. Genes with two or more missing values were removed from further analysis. Missing values were possibly due to a bad signal to noise ratio. A gene was considered to be differentially expressed when the mean value of the ratio log2 (Cy5/Cy3) was > 1.58 or < -1.58 and the cDNA was identified with a one class significance analysis of microarrays (based on SAM (180)) with a False discovery rate

Table 1. Primers and RT-PCR conditions for different genes.

Gene	Forward primer	Reverse primer	Annealing temperature
XM_416896 (lysozyme G)	CGGCTTCAGAGAAGATTG	GTACCGTTTGTCAACCTGC	62°C
XM_416085 (carboxypeptidase M)	ATTCTGGAGAGACAACAAAGTTGCT	TTTGGCTTCCACGATTGCA	58°C
XM_425603 (ORF2)	GTCAGCCTCTTCTCTCGTGTGA	AGTGCCTGACCACCCTTTCA	58°C
BX930518 (clone ChEST640b17)	GAATCAAGCAACTTCCGTACCAT	AGGTTCCAAGAGCCTGAAAGTTC	59°C
XM_420282 (DNA segment, Chr 10)	TCTTCCCAGGCTGTGAG	GGTCACCAGCTTGTTCTTC	64°C
NM_205513 (calbindin)	CATGGATGGGAAGGAGC	GCTGCTGGCACCTAAAG	56°C
DQ_018756 (28S)	TCAACTTTCCCTTACGGTAC	CAAGTCCTTCTGATCGAG	56°C

< 2%. Because the ratio was expressed in a log2 scale, a ratio of > 1.58 or < -1.58 corresponded to a more than threefold up- or downregulation respectively, which is the expression difference limit indicated by the manufacturer of the MICROMAX TSA labelling and detection kit (PerkinElmer, Wellesley, MA).

Quantitative real time PCR

A quantitative PCR was performed as described previously (184). Briefly 200 ng of total RNA from the jejunum was reverse transcribed with random hexamers. Generated cDNA was stored at –20°C until use. PCR amplification and analysis was done with the described primers and conditions (Table 1). 28S was used as a control to correct for the input of cDNA. Each reaction mixture consisted of 1 µl cDNA (1:10 diluted), 1 µl of each primer (10 µM solution), 2 µl LightCycler FastStart DNA Master SYBR Green mix, 3 mM $MgCl_2$ in a total volume of 20 µl. All templates were amplified with a preincubation for 10 minutes at 95°C followed by amplification for 40 cycles: (5 sec 95°C, 10 s annealing temperature, 15 s 72°C).
In each run, four standards of the gene of interest were included with appropriate dilutions of the DNA, to determine the cDNA concentration in the samples. All RT-PCRs amplified a single product as determined by melting curve analysis. To see if the groups differed significantly, a student-t test was performed on the log transformed

concentrations corrected for the amount of 28S and $p<0.05$ was considered as significant.

Results

Bacteriological examination and body weight

In all the animals inoculated with *Salmonella enterica* serovar Enteritidis the *Salmonella* was detected in the faeces at all time points analysed by bacterial platings. In contrast, in none of the control animal *S. enterica* serovar Enteritidis was detected. The number of *S. enterica* serovar Enteritidis found in the liver of chickens from the fast growing (F) and slow growing (S) line is presented in Figure 6.1. At day 1 and 5 pi the chickens from line S had more cfu *Salmonella* in the liver compared to line F ($p<0.05$).

As Line F is the fast growing chicken line, from day 5 onwards the healthy chickens from this line were heavier than the healthy chickens from line S ($p<0.001$). The chickens from line S had a weight gain depression due to the *Salmonella* infection ($p<0.01$) while the chickens from line F had no significant weight gain depression after the *Salmonella* infection (Figure 6.2).

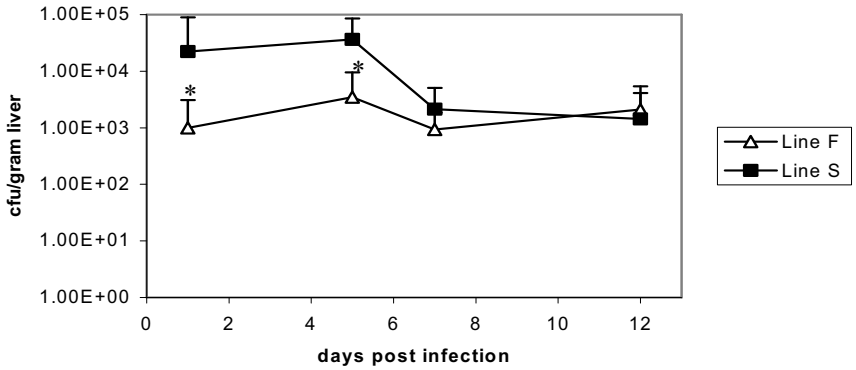

Figure 6.1. Number of CFU of *S. enteritidis* in the liver of chickens from the two chicken lines. Error bars indicate the SEM (n=10)* Infected chickens from line F have significant less cfu *Salmonella* in the liver than chickens from line S ($p<0.05$).

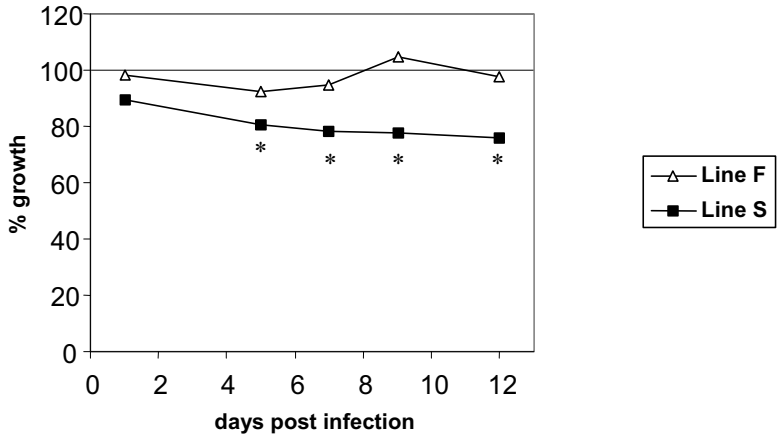

Figure 6.2. Percentage growth of chickens infected with 10^5 *S.* Enteritidis compared to healthy counterparts. * Infected chickens are significant lighter than age-matched healthy counterparts from the same line ($p<0.05$).

Phagocytic activity and flow cytometric analysis of intestinal cells

The phagocytic activity of the isolated intestinal mononuclear cells was measured with the phagocyte assay and the results are shown in Table II. However, at least 1×10^7 mononuclear cells per chicken were necessary for this assay and not from all jejunums enough cells were isolated. Therefore for this assay 3 to 10 animals per group were used. The isolated intestinal mononuclear cells were stained with trypan blue to check the viability of the cells and were analyzed with FACS for the percentage of $CD45^+$ cells. The gated mononuclear $CD45^+$ cells in the jejunum increased with age of the chickens (Figure 6.3). At day 1 pi the *Salmonella* infected chickens from line F had a significant lower percentage $CD45^+$ cells compared to their healthy counterparts ($p<0.01$).

For the phagocytic activity only effects within each day can be compared, due to the differences in number of bacteria between the overnight *Salmonella* cultures for the different days pi. At day 1 pi the cells isolated from the *Salmonella* infected chickens from line F had almost 7 times more bacteria internalised compared with the cells from the control chickens ($p<0.01$). In contrast in line S no differences between the cells of the control chickens and the *Salmonella* infected chickens were found at day 1 pi (table 2). However, at day 5 pi the cells from the *Salmonella* infected chickens from line S

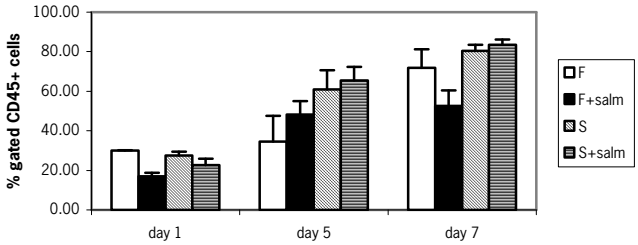

Figure 6.3. Amount of $CD45^+$ cells in the isolated intestinal mononuclear cells as a percentage of the gated intestinal mononuclear cells. F= fast growing chicken line, S = slow growing chicken line, + salm = infected with *Salmonella enteritidis*. Standard bars indicate the SEM (n= 3-10).* Significant difference between cells from control and infected chickens ($p<0.01$).

internalised two times more bacteria than the cells from the control chickens from the same line ($p<0.05$). At day 5 pi in line F no differences in the amount of internalised bacteria between the control and the *Salmonella* infected chickens were found (Table 2). At day 7 pi in neither chicken line differences in phagocytic activity were found between cells from the *Salmonella* infected and the control chickens.

Immunohistochemistry

Frozen jejunum sections from all animals were quantified for CD4 positive T-cells, CD8 positive T-cells and macrophages with immunohistochemistry and we found small but significant differences between the groups (Table 3). The number of $CD4^+$ T-cells per mm^2 was at day 1 and day 5 pi lower in the *Salmonella* infected chickens from line F compared to the healthy age-matched controls from the same line. There were no clear differences in the location of the $CD4^+$ T-cells, as most cells were located in the

Table 2. Phagocytic activity of mononuclear gut cells of chickens[a].

Line	Day 1		Day 5		Day 7	
	control	infected	control	infected	control	infected
F	9 (± 5)[b]	64 (± 9)	5 (± 1)	5 (± 4)	357 (± 26)	364 (± 32)
S	33 (± 10)	48 (± 8)	7 (± 1)[b]	16 (± 4)	378 (± 33)	396 (± 33)

[a] Total number × 10^3 of internalised bacteria by all cells ± SEM.

[b] Significant difference between control and *Salmonella* infected chickens ($p<0.05$).

lamina propria. For line S no differences in the amount or location of $CD4^+$ T-cells was found between the control and the infected chickens. The number of $CD8^+$ T-cells was increased at day 7 pi in the infected chickens from line S compared to their controls and at day 5 and 7 pi for the infected chickens from line F.

At day 1 pi the number of macrophages in the *Salmonella* infected chickens from line S was lower than the healthy chickens from line S or the *Salmonella* infected chickens from line F. At later timepoints no significant differences between the amounts of macrophages were found.

Gene expression in the chicken intestine

RNA was isolated from the chicken jejunum day 1 pi to investigate gene expression responses to *Salmonella*. Comparing the gene expression responses of both chicken lines we found more differences than similarities 1 day post infection. After the *Salmonella* infection three genes were more than threefold upregulated and six genes were more than threefold downregulated in line S, but not in line F (Table 4). In line F liver fatty acid binding protein was downregulated after the infection, whereas no significant regulation was observed in line S.

In addition to the genes regulated after the *Salmonella* infection in one of the chicken lines, some genes were regulated in both chicken lines. Similar to DNA segment, Chr 10, ras homolog gene family member T1, dickkopf homolog 3, similar to fatty acid synthase and cytidine deaminase were upregulated, whereas calbindin was downregulated more than threefold in both chicken lines (Table 4).

Table 3. Mean number (± SEM) of $CD4^+$, $CD8^+$ and macrophages per mm^2.

	day 1		day 5		day 7	
Line	control	infected	control	infected	control	infected
$CD4^+$ T-cells						
F	14 (± 2) [a]	8 (± 1)	45 (± 3)[a]	33 (± 3)	100 (± 4)	113 (± 7)
S	12 (± 2)	16 (± 2)	68 (± 5)	54 (± 8)	74 (± 7)	91 (± 5)
$CD8^+$ T-cells						
F	38 (± 4)	57 (± 8)	329 (± 27)	300 (± 29)	211 (± 21)[a]	335 (± 24)
S	32 (± 6)	26 (± 3)	168 (± 11)[a]	237 (± 24)	193 (± 15)[a]	254 (± 19)
macrophages						
F	226 (± 14)	241 (± 17)	353 (± 16)	382 (± 19)	451 (± 44)	485 (± 36)
S	213 (± 14)[a]	124 (± 8)	391 (± 30)	395 (± 25)	469 (± 25)	457 (± 16)

[a] Significant difference between control and *Salmonella* infected chickens ($p<0.05$).

Table 4. Fold changes in mRNA compared with age-matched mock-infected controls 24 hours after the *Salmonella* infection.

Accession no	Gene name	Foldchange (infected/ control)	
		Line F	Line S
Genes regulated in line S after *Salmonella* infection			
XM_416896.1	PREDICTED: Gallus gallus similar to lysozyme G	1.0	6.9
CR522945	finished cDNA, clone ChEST753p12	1.3	4.2
XM_418587	PREDICTED: Gallus gallus similar to CG3524-PA (LOC420485),	1.5	3.1
DN828701	expressed sequence tag (Chr:2 80621800 - 80622343)	-1.7	-27.4
XM_416085.1	PREDICTED: Gallus gallus similar to Carboxypeptidase M precursor (LOC417843), mRNA	-2.0	-7.2
XM_425603.1	PREDICTED: Gallus gallus similar to ORF2 (LOC428036), mRNA	-2.3	-6.1
XM_423002.1	similar to Rho GTPase-activating protein; brain-specific Rho GTP-ase-activating protein	-1.8	-4.7
BX930518.1	Gallus gallus finished cDNA, clone ChEST640b17	-1.0	-3,5
BU457068.1	cDNA clone ChEST200c16	-1.2	-3.3
Genes regulated in line F after *Salmonella* infection			
NM_204192.1	Gallus gallus fatty acid binding protein 1, liver	-4.0	-1.1
Genes regulated after a *Salmonella* infection in both chicken lines			
XM_420282.1	PREDICTED: Gallus gallus similar to DNA segment, Chr 10, Johns Hopkins University 81 expressed	4.8	19.1
NM_001006208	Gallus gallus ras homolog gene family, member T1 (RHOT1)	7.9	15.0
NM_205125.1	Gallus gallus dickkopf homolog 3	4.7	14.2
XM_418586	PREDICTED: Gallus gallus similar to Fatty acid synthase	3.7	6.0
NM_204933.1	Gallus gallus cytidine deaminase (CDD)	2.9	3.6
NM_205513.1	Gallus gallus calbindin 1, 28kDa	-3,2	-3,8

Table 5. Expression differences found with the microarray compared with the q-PCR.

	Line F Ratio salmonella/control[a]		**Line S** Ratio salmonella/control[a]	
Gene	microarray	q-PCR	microarray	q-PCR
XM_416896 (lysozyme G)	1.0	-1.0	6.9	14.0[b]
XM_416085 (carboxypeptidase M)	-2.0	-1.1	-7.2	-3.2 [b]
XM_425603(ORF2)	-2.3	-1.3	-6.1	-2.4 [b]
BX930518 (clone ChEST640b17)	-1.0	-1.1	-3.5	-3.0[b]
XM_420282 ((DNA segment, Chr 10)	4.8	3.9 [b]	19.1	14.1 [b]
NM_205513(calbindin)	-3.2	-2.6 [b]	-3.8	-2.8 [b]

[a] When the ratio (salmonella/control) is smaller than 1, the ratio –(control/salmonella) is given.
[b] The expression levels of the control and Salmonella infected group from the same chicken line differ significantly (student T-test, $p<0.05$).

For lysozyme G, carboxypeptidase M, similar to ORF2, cDNA clone ChEST640b17,similar to DNA segment, Chr 10 and calbindin a quantitative PCR was performed on the individual samples. For all these genes the up- or downregulation we found with the microarray was confirmed with the RT-PCR. Furthermore when more than threefold expression differences were detected with the microarray, which was our threshold to call a gene up- or downregulated, the expression levels differed significantly ($p<0.05$) between the control and the *Salmonella* infected chickens from the same line (Table 5).

Discussion

Salmonella bacteria that reach the intestinal tract can cross the intestinal epithelium after attachment to the mucosa. From there, they can reach the lamina propria, where they replicate or penetrate to deeper tissues. After reaching the blood stream, they infect internal organs, such as liver and spleen. In our experiment, colonisation of the liver started already 1 day post inoculation, with 40 and 90% of the chickens positive from line F and S respectively. It was unexpected that the chickens from line F had no body weight loss, because it was suggested that fast growing meat-type chickens are more susceptible to *Salmonella* compared with slow growing ones (92). On the contrary it has also been reported that meat-type chickens, which grow

fast, are more resistant to *Salmonella* compared with laying-type lines (62). So overall the relation between *Salmonella* susceptibility and growth rate is not unambiguous. Nevertheless the two chicken lines used in this experiment showed a clear difference in outcome of a *Salmonella* infection and it was interesting to further analyse their intestinal responses to the *Salmonella* infection and to compare gene expression between these lines and lines that were used in an earlier study (185).

At day 1 pi the isolated intestinal mononuclear cells from the *Salmonella* infected chickens from line F had a higher phagocytic activity than the control animals. These differences were not due to differences in the amount of macrophages as determined by immunohistochemistry. Possibly the macrophages of the infected animals are more active. When the macrophages are more active, less *Salmonella* bacteria are able to survive. Indeed the number of cfu *Salmonella* in the liver of the infected animals from line F were significantly lower than in the infected chickens from line S. Another option is that the isolated cell suspension of the infected animals from line F are of a different composition compared with the cell suspension of the control animals. The percentage of macrophages in the isolated cell population can differ between infected and control animals causing the difference in the number in phagocytosed bacteria. The infected animals had a lower percentage of $CD45^+$ cells (marker for leukocytes) in the isolated cell population, thus other non $CD45^+$ cells were used in our phagocytose assay, because the amount of mononuclear cells used in the assay was equal among the groups. Thus either the macrophages are more active or other cells are responsible for a better phagocytose activity in the cell population isolated from the *Salmonella* infected animals.

At day 5 pi differences in the phagocytic activity between the intestinal mononuclear cells between the infected and the control chickens were found for line S instead of line F as for day 1 pi. This was also not caused by differences in the amount of macrophages. Here no differences in amount of $CD45^+$ cells in the isolated cell populations were found. At day 7 no differences in phagocytic activity of the intestinal mononuclear cells were found, which is in agreement with an earlier study (91). Unfortunately in that study no earlier timepoints than day 7 pi were investigated. So we found at early timepoints post infection differences in the phagocytic activity of the mononuclear cells from the intestine of control and *Salmonella* infected animals, but the causes of these differences are not known.

The number of $CD4^+$ was decreased in line F in response to the *Salmonella* infection, but not in line S. This is surprising because in most studies an increase in the amount of $CD4^+$ T-cells in the ceca was found after a *Salmonella* infection (8, 19). Also in mice the number of $CD4^+$ T-cells increased in the gut following *Salmonella* challenge (12, 120). Furthermore in the ovary and oviduct of laying hens increased numbers of $CD4^+$ T-cells were reported after a *Salmonella* infection (14, 197, 198). However, it for the early host response $CD4^+$ or $CD8^+$ T-cells are probably not required (112). In addition here young chickens were investigated where the intestinal immune system is immature, which could be reason that no increase in $CD4^+$ T-cells was found.

For $CD8^+$ cells we found an increase after the *Salmonella* infection in both chicken lines, but faster in time post infection in line F. An increase in the amount of $CD8^+$ cells was also found in the ceca after a *Salmonella* infection (8, 19), but also decreases in the amount of CD8+ cells in the ceca are reported (158). In the oviduct of laying the numbers of $CD8^+$ cells were increased after a *Salmonella* infection (14, 197, 198). Our and other results suggest that differences in influx of T-cell subpopulations after a *Salmonella* infection are dependent on the location in the digestive tract, infection dose, time post infection, age at the time of infection and genetic background of the chickens.

The gene expression as measured with the microarray and validated with quantitative PCR in the jejunum at day 1 pi in these two chicken lines was partial in correspondence with our earlier studie (185). Besides differences in gene expression responses between the two chicken lines we saw again upregulation in both lines of cytidine deaminase, similar to fatty acid synthase, dickkopf homolog 3 and similar to DNA segment, Chr 10, Johns Hopkins University 81 expressed in response to the *Salmonella* infection. It was noteworthy that al these genes were more upregulated in line S, which had higher growth retardation and more cfu in the liver after the *Salmonella* infection than line F. However, in earlier experiments no relation was found with severity of the systemic infection and level of upregulation (185). So these four genes are upregulated during a *Salmonella* infection, irrespective of the severity of the systemic infection and the growth retardation.

Some of the downregulated genes due to the *Salmonella* infection in line S might be related to decreased amounts of macrophages, as was shown by in situ hybridisation. One of the downregulated genes is carboxypeptidase M, a macrophage differentiation marker (144). Also similar to ORF2 (LOC428036) was downregulated, whereas this gene was shown before to be upregulated in avian macrophages after

phagocytosis of Escherichia coli (24), so lower expression levels of this genes might indicate a decrease in the amount of macrophages.

This is the first report about changes in T-cell subpopulations and macrophages in the chicken jejunum in response to a *Salmonella* infection in one day old broilers and these changes were different between the two lines used. We also found gene expression differences between the two chicken lines, so the genetic background of the chickens is important for their responses to a *Salmonella* infection.

Chapter 7.

General discussion

Main findings

Chicken lines display hereditary differences in susceptibility to various diseases, like Malabsorption syndrome (MAS) and a *Salmonella enteritidis* infection. The main aim of the studies described in this thesis was to identify genes and pathways involved in genetic disease resistance, and to get insight into the mechanisms that determine differences in resistance to infectious diseases. For this purpose gene expression was examined in the intestine of chicken lines with a genomics approach using cDNA microarrays. Gene expression profiles in response to MAS and *S. enteritidis* were investigated as well as differences in gene expression levels between chicken lines under control and challenge conditions. Three main findings were obtained during the studies described in this thesis. Firstly, a number of genes has been identified that are differentially induced between chicken lines after challenge. Secondly, it was found that most changes in gene expression in response to an infection were found very early after exposure to the infectious agent. The third finding was that chickens with different genetic backgrounds had large differences in gene expression profiles after infection, more genes were differently regulated between the lines than that there were similar regulation patterns. These three findings will be discussed below more extensively.

Disease resistance genes

Genes were identified that are regulated in response to an infection in one chicken line, but not in another chicken line. This suggests that these genes are somehow involved in determining the difference in the susceptibility trait of these lines. Based on this, several pathways and processes involved in disease susceptibility have been identified. After MAS induction immune related genes and genes associated with feed absorption were differentially expressed between the chicken lines, as were genes with unknown functions. After the salmonella challenge the differentially expressed genes between the chicken lines were genes involved in processes like T-cell activation, macrophage activation, inflammation, acute phase responses, actin polymerisation, and the fibrinogen system. In addition to the “challenge-specific response” genes, also some “common response” genes were identified. These “common-response” genes differed in expression regulation between susceptible and resistant animals in response to both MAS induction and a salmonella infection. In both cases cytochrome P450 (chapter 3 and 4) and apolipoprotein B (chapter 3 and 4) were down-regulated in the susceptible chicken lines and not in the resistant chicken lines. In addition, lysozyme G (chapter3 and 6) was up-regulated in the susceptible chicken lines and not in the

resistant chicken lines. Each of the proteins cytochrome P450, apolipoprotein B and lysozyme G are involved in early defence mechanisms. Lysozyme G is a cationic antimicrobial protein (130) and apolipoprotein B is a negative acute phase protein (102). Both cytochrome P450 and apolipoprotein B are down-regulated in response to proinflammatory cytokines (21, 189).

Further evaluation of the expression levels of cytochrome P450, apolipoprotein B and lysozyme G will reveal their potential use as general biomarkers for gut health and susceptibility to intestinal diseases. To take this one step further, more chicken lines and other infection models should be investigated, for instance infections with different bacterial pathogens (gram-positive and gram-negative), viruses or parasites. It will also be interesting to investigate the expression of these genes in other organs than the intestine, like lung, liver and spleen and to examine serum concentrations in blood for the encoded proteins. Serum levels can easily be measured, whereas for the examination of gene expression levels in organs a biopsy must be taken or the animal must be killed. Furthermore, it would be interesting to investigate whether there exists a correlation between mRNA expression- or protein concentration levels of these genes or geneproducts with the intestinal health status of the animal, in order to obtain biomarkers for intestinal health. In addition it can be investigated whether these genes are also regulated in other species than chicken in response to an infection. Cytochrome P450 and apolipoprotein B are well known mammalian genes, but why their intestinal expression is regulated in response to an infection is unclear. For apolipoprotein B it was shown that serum concentrations are decreased in individuals with Crohn disease, an inflammatory bowel disease (99). It is also known that the expression of cytochrome P450 in the liver is modified during infection or inflammation (146). Expression of lysozyme G has previously been shown in different organs in various birds and in various fish species (70, 130, 205), but the expression of lysozyme G has not been studied yet in the context of challenge experiments.

To our surprise almost no known immune related genes, like chemokines and cytokines, were found to be regulated in the intestine in response to the infections. This might be due to the absence of expression differences of these immune genes at the investigated time points or to low expression levels in the investigated biological samples. The inability to detect chemokine and cytokine induction may also be due to the absence of these genes on the home-made array. However, also with the whole genome Affymetrix array (chapter 5), only K60 and IL-8 were found to be up-regulated in only one chicken line among the many other genes that were differently

expressed in the two chicken lines. This emphasizes that a focus only on chemokines and/or cytokines ignores important other gene expression levels and cellular processes altered in the gut early after infection.

Major differences early after infection

In the studies described in this thesis, most changes in gene expression in response to an infection were found 24 hours after exposure to the infectious agents. Three days post infection most of these differences were no longer present, indicating that the time-points chosen are crucial to gain insight in host response mechanisms. These findings indicate that if processes at the onset of a disease are studied, early time-points post infection should be investigated. In the MAS experiment gene expression was studied 8 hr after disease induction, but at that timepoint almost no expression differences were found between infected and control animals. This is in accordance with the onset of pathological changes after MAS induction. Vacuolar degeneration of villus surface epithelial cells and erosions of the tip of villus are observed at day 1 post inoculation and also the influx of heterophils into the lamina propria appear from day 1 pi (210). Also the expression of different cytokines as measured with quantitative PCR was not altered 8 hours pi, whereas the expression of IL-2, IL-6, IL-8 and interferon-γ was found to be up-regulated at later time-points post the MAS induction (139). In the salmonella experiments 24 hours pi was the first timepoint examined for gene expression in our studies. After a salmonella infection in young birds microscopically changes in the intestine are seen from 24 hr pi, whereas CXC chemokine K60 and IL-8 are already up-regulated 12 hours pi (196), indicating that gene expression changes precede pathological changes.

Selection for immune responsiveness at the level of antibodies and T-cell populations is still regarded as a potential approach to improve disease resistance in animals. Several days are required for the clonal expansion and differentiation of naive lymphocytes into effector T-cells and antibody secreting B-cells (76). The studies described in this thesis clearly demonstrate that major host responses are found 24 hours post infection. This suggests that also innate immune mechanisms are likely to be important in determining intestinal disease susceptibility. Therefore, an alternative for the selection of chicken lines with an improved disease resistance would be to focus more on innate immune mechanisms instead of the adaptive mechanisms. It will be interesting to investigate whether innate immune parameters, like macrophage or heterophil activity or early gene expression profiles, correlate with disease resistance.

Here it was shown (chapter 6) that chickens with differences in salmonella susceptibility also differ in the phagocytic properties of their intestinal mononuclear cells. In addition, a decrease in the amount of macrophages in the susceptible chicken line correlated with a down-regulation of the expression of certain genes. However, caution must be considered to extrapolate phagocytic data to the *in vivo* situation, as high heterophil activity might not always be beneficial for reduction of disease development. When heterphils have higher activity, more nitric oxide will be produced, causing tissue damage.

Genetic background determines nature of host response

One of the most surprising findings of the studies described here, was that the genetic background of chickens influences the nature of the gene expression response to an infection to a very large extend. In fact, more differences than similarities were found between the different chicken lines in response to the infections. Generally it is thought that there is a common host transcriptional response to pathogens (77). Therefore many genes were expected to be regulated in a similar way in all chicken lines. In the various animal experiments and challenge models that were used no single gene was identified that was similarly regulated in all lines in response to the challenges. One reason for this might be that not the same set of genes was studied in all the experiments. For example most of the identified genes described in chapter 4 and 6 were derived from a spleen library, whereas the clones of this library were not present on the microarray used in the experiments described in chapter 3. The genes described in chapter 5 were identified with the aid of the Affymetrix microarray and only a subset of these genes is present on the microarrays described in the other chapters. In addition, only the samples taken at 1 day pi were investigated in the studies described in chapters 5 and 6, whereas the more common host response may take place at another stage after infection. Another reason that we found more differences in gene expression than common host responses between the chicken lines early after challenge, might be due to the fact that here *in vivo* gene expression profiles were examined. These profiles represent the expression pattern of a heterogeneous cell population. In contrast, most of the studies, where common host transcriptional programs to pathogens were identified, have been performed *in vitro* using homogeneous cell populations. Some aspects about *in vivo* versus *in vitro* studies will be discussed further in the paragraph dealing with the methodological aspects of the experiments. To study genetic causes of disease susceptibility, two genetically different chicken lines were compared for each of the experiments described in the chapters 3-6.

Unfortunately, not in all experiments the same chicken lines were used. In all cases the chicken lines were obtained from commercial breeders. The compared chicken lines were genetically unrelated, nor they were inbred lines divergently selected from a common ancestral line. The chicken lines with different susceptibilities to MAS were described by Zekerias *et al*. (209). In contrast, no well described chicken lines with differences in salmonella susceptibility were available. However, it was suggested earlier that chicken lines with differences in growth rates might also show differences in salmonella susceptibility (92). Therefore a slow growing broiler line was compared to one of the chicken lines used in the MAS experiment. As already indicated above, by comparing these two chicken lines, gene expression differences related to differences in salmonella susceptibility could be identified. However, the observed gene expression differences could also be due to differences in growth or development. To overcome this problem we focussed the analysis on genes which met the following two criteria: their expression was altered in response to the infection in only one of the two chicken lines and their expression differed between the chicken lines either in the control situation, or the infected situation (Figure 7.1). Nevertheless, it would have been better to compare two chicken lines with the same growth rate that differed in salmonella susceptibility. Unfortunately such lines were not available.

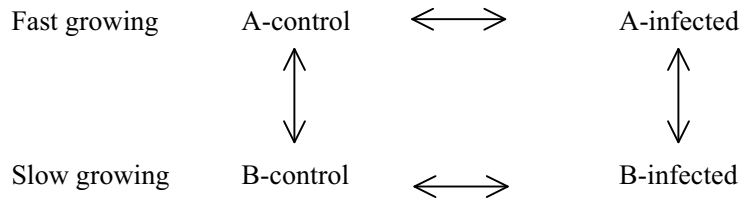

Figure 7.1. For a single timepoint four comparisons were performed. Analysis was focussed on genes differentially expressed in at least two of these comparisons (one between the two chicken lines, vertical arrows, and one between control and infected animals, horizontal arrows).

As already indicated, the results clearly demonstrated that different chicken lines display quite different expression profiles in response to an infection. This suggests that also the biological response to an infection differs between chicken lines, some of these responses have been described in chapter 6. This suggestion is also supported by previous observations by others. Fundamental immunological differences between broiler and layer chickens are well established (88, 98). Peripheral blood leukocyte

populations differ significantly between chicken lines in the percentage of positive CD3 T-cells, the ratio of B-cells: T-cells, and CD3 and CD8 T-cell receptor density (36). Immunological gene expression was studied and it was shown that layers had a higher level of mRNA expression than broilers for CXCLi2, IL-10, IL12α and CCLi2 in the spleen, but in the cecum the layers had lower mRNA expression levels than the broilers for IL12α, IL12β and CCLi2 (35). Expression differences were also detected in splenocytes after Marek's disease virus infection between susceptible and resistant animals for IL-6 and IL-18 (82). Also under control conditions gene expression levels differed between different chicken breeds (chapter 3 and 4). Hence, it is likely not always possible to generalize the research results obtained with a particular chicken line. The great variation in gene expression between chicken lines might explain a part of the problems encountered in the comparison of research results of different research groups.

Host gene expression responses to an infection are often investigated in a single breed of animals with the idea that the responses are of general nature. Apparently this is a wrong idea as we found almost no common responses between genetically different animals, at least early after infection. It has been described earlier that immune responses are under genetic control (11, 117), and here it was shown that also broader gene expression responses are under genetic control. Overall, only limited information is available about genetic variance in host gene expression responses to an infection, while this is probably as important as genetic variance in pathogenic strains.

Methodological aspects

During the study described in this thesis insights in methodological issues have been obtained that might be useful to take into account for further studies on intestinal health and intestinal disease susceptibility. First several aspects about the use of a genomics approach will be discussed followed by a discussion on the choice for *in vivo* or *in vitro* experiments. Finally, some methods for functional validation of differentially expressed genes will be described.

Microarrays

In the experiments described in this thesis microarrays were used to study gene expression differences, because microarrays allow the analysis of the expression of thousands of genes simultaneously. It is an unbiased approach where no genes or proteins are chosen beforehand, as is the case with other approaches like northern blotting, quantitative PCR, *in situ* hybridisation and immunohistochemistry. With a

microarray it is possible to identify new genes involved in the investigated trait. Another advantage of such a functional genomics approach is that up- or down-regulated genes are part of the biological pathways involved in the trait under study. Therefore analysis of microarray data gives more physiological relevant information than other genomics techniques like Quantitative Trait Loci (QTL) and Single Nucleotide Polymorphisms (SNPs).

For the experiments described in chapters 2 to 6, we used in-house produced microarrays that contained between 3000 and 4300 genes, which were obtained from subtracted and normalized cDNA libraries. At the time this study began, this was the state of the art of this technology in chicken. At present these arrays are considered to be small and incomplete. The currently available whole chicken genome microarray from Affymetrix for example contains almost 33,000 probe sets representing over 28,000 (predicted) chicken genes. Also a whole genome array with 20,000 oligo-nucleotides representing 20,000 (predicted) genes has recently been developed for chicken. Despite the limitation of the used microarray in our studies, insight has been obtained in new processes that are affected in the intestine by infectious diseases.

The microarray data described in chapter 3, 4 and 6 were verified with quantitative PCR (qPCR) analyses. With this technique individual samples can be tested for their expression levels of specified genes whereas for the microarrays pools of samples were used. In all cases the qPCR data very well confirmed the microarray data and the gene expression differences between the groups were significant for most genes when tested individually. This is likely due to the use of a relatively high threshold during the analysis of the microarray data and to low variations between individuals of the same group. Genes were called up- or down-regulated only when the expression differences were higher than a certain threshold, like 3 or 4 times. The advantage of this approach is that the percentage of false positive signals is low, the disadvantage is that we could have missed some genes with small but significant differences in up- or down-regulation. When one is interested in small expression differences, individual samples instead of pools should be used in the experiment. With individual samples hybridised on the microarray, more sophisticated statistical methods can be used to evaluate whether a gene is up- or down-regulated.

In our studies a large number of genes with unknown functions have been identified that are involved in the response to an infection. We were not able to link these genes to biological pathways or cellular processes due to the lack of data analysis and data interpretation tools and the limited annotation and gene ontology terms currently assigned to chicken genes. Data analysis and interpretation tools need to be

further developed to facilitate data interpretation. Further effort should be undertaken to finish the sequencing and assembly of the whole chicken genome and to provide fuller annotations. Fuller annotations will facilitate data analysis as better comparison with known pathways in other animal species will be possible. Annotation may be based on conserved gene functions across species, but must rely on new evidences if there is a low level of sequence conservation. For several groups of genes there is a low level of sequence conservation between birds and mammals, especially the immune related genes are evolutionarily not well conserved (80). In addition, different synonyms have been used for a single gene within and between species, which complicates automatic annotation. Whole organ proteomics might help to assign new annotations to chicken genes. Such an approach has recently been applied for the avian bursa of Fabricius to further annotate the chicken genome (114).

In vivo* versus *in vitro

So far, most of the studies focussing on host gene expression responses upon exposure to micro-organisms have been performed *in vitro* (77). For example, it was shown that upon *in vitro* treatment with *S. enteritidis,* heterophils from salmonella resistant chickens had higher levels of pro-inflammatoy cytokine mRNA compared to heterophils from salmonella susceptible chicken lines (178). For the studies described in this thesis gene expression was studied *in vivo.* Large differences can be expected between the gene expression profile of cell culture systems and tissues derived from living organisms. Cell lines are placed outside their normal environment, and contact with other cell types and specific environmental conditions is lacking (155). In addition, cell lines are often cultured for long periods which may have changed their behaviour (9, 40). Furthermore, due to absence of growth inhibition, the cell lines do not display the physiology of normal cells (9, 51). Additionally, a cell culture system usually exist of only one cell type, whereas *in vivo* different cell types act and interact together. The intestine is a complex system with a continuous cross-talk among epithelial cells, the local immune system and the microflora in the lumen, which cannot be easily mimicked *in vitro* (9, 155).

A disadvantage of *in vivo* experiments is the complex interpretation of the data that are obtained (105). For all the gene expression profiles investigated in the studies described in this thesis, the whole intestine was used instead of only the epithelial cells. The main reason was a practical one, as the intestine of young chickens is too small to obtain sufficient cells with mucosal scrapings. Furthermore the whole intestine could be snap frozen in liquid nitrogen immediately after section, resulting in good quality of

RNA. A disadvantage of the use of the whole intestine is the complex picture of the data due to the presence of different cell types, which makes interpretation more difficult. An important or local gene expression pattern might be undetectable amidst a "noisy" environment, particularly a pattern generated by a rare cell population. It can be expected that different cell types regulate some common as well as some specific genes in response to an infection (77). For example some cytokine genes are more strongly induced in peripheral blood mononuclear cells compared to other cell types such as macrophages, dendritic cells and epithelial cells (77). In addition, when a gene is identified as upregulated or downregulated in a microarray experiment using tissue samples from *in viv*o experiments, this might not always be real regulation at the level of transcription, but might also be caused by the influx of other cell types, like heterophils or lymphocytes in the tissue samples. Increase or decrease in the relative abundance of a cell type alters the overall proportion of unique transcripts from that cell type in the total pool of RNA from a given sample. One way to overcome this problem is the use of laser micro-dissection. With this technique specific cell types can be isolated from microscopic slides. Consecutively RNA can be isolated and used to investigate their gene expression profile (133). With this technique it has for example been shown that a *Helicobacter pylori*-specific transcription profile is induced only in the mucus-producing pit cells from marine stomach tissue (125).

Functional validation

The gene expression results of the first salmonella experiment (described in chapter 4) suggested differences between the chicken lines in T-cells activation or maturation and the number or activation of macrophages. Therefore in the second salmonella experiment the number of $CD4^+$ and $CD8^+$ T-cells in the jejunum was counted as was the number of macrophages in the intestine. Also the gene expression profiles were investigated. The T-cell related genes that were induced after a salmonella infection in the first experiment showed no expression differences in the second experiment. However, the down-regulation of carboxypeptidase M and a gene "similar to ORF2 (LOC428036)" in one chicken line might be correlated to the decrease in the amounts of macrophages that was found in the intestine of the same chicken line.

To unravel the physiological role of differentially expressed genes in disease susceptibility, functional tests should be developed. *In vitro* models would be useful to evaluate the function of genes identified in the studies described in this thesis. Unfortunately at present no *in vitro* model for the chicken intestine is available. An

intestinal cell line has been described in literature (188), but these cells are no longer available (Philip Velge, personal communication). Hence for further evaluation of the function of genes in the intestine the generation of a chicken intestinal cell line will be valuable.

To evaluate the function of a number of genes *in vitro*, during this research period a lot of efforts were undertaken to clone these genes into an expression-vector. With these constructs multiple cell lines were transfected. The cell lines used were the macrophage cell lines NCSU and HD11, a T-cell line (MSB-1), a B-cell line (RP9) and a fibroblast cell line (DF1) from chicken origin and a colon epithelium cell line (HT29) from human origin. A number of difficulties were encountered: genes which were unable to be cloned, cell lines which were unable to be transfected and transfected cell lines grew very slow or were not stably transfected. The ikaros transcription factor (chapter 4, upregulated after salmonella infection in susceptible chicken line) was overexpressed in the HD11 and DF1 cell lines and multiple assays were performed. Ikaros is a DNA-binding protein that is required for lymphocyte development (39). Overexpression of ikaros caused no differences in phagocytosis of salmonella bacteria by the HD11 and DF1 cell lines. Both HD11 and ikaros-transfected HD11 cell lines upregulated IL-12 when salmonella bacteria were added to the cells, but the wild-type HD11 cells had higher expression of IL-12 compared to the ikaros-transfected HD11 cells. In the DF1 cell lines we did not observed differences in IL-12 expression between the wild-type and the ikaros-transfected cells. We also examined the possibility of salmonella migration through the cell-layer of wild-type and ikaros-transfected HD11 and DF1 cell lines, but it was difficult to obtain monolayers of the cells which were necessary in the test-system setup and, consequently, the results were not unambiguous. However there were some suggestions that the ikaros-transfected HD11 cell line had a lower permeability for the salmonella bacteria than the wild-type HD11 cell line. This description demonstrates only some of the problems we encountered to evaluate *in vitro* the functions of genes.

Possibly overexpression in cell-lines is not the most appropriate approach to study the function of genes. A specific gene knockdown might be valuable to study individual genes to learn more about their function, their biological relevance and their role in disease susceptibility. A specific gene knockdown can be obtained by application of the RNAi technology. This is an emerging technology to study gene function. Gene silencing by RNAi is typically incomplete, a gene knockdown rather than a gene knockout will be achieved. A few studies have demonstrated *in vivo* silencing using RNAi. For example, in mice the intravenous administration of RNAi

specific for the gene encoding the Fas receptor prevented the animals against liver damage caused by hepatitis (170). Since in chicken some RNAi approaches have already been described in the cell line DF1 as well as in embryos (123, 127), there is great potential of RNAi for functional genomic studies in chickens.

To identify the pathways involved in genetic differences in salmonella susceptibility, an automated pathway analysis was performed using the dataset obtained with the Affymetrix array (described in chapter 5) and the KEGG database (83). The microarray contained 32,773 *Gallus gallus* transcripts, of which 3520 gene-pathway combinations were found in the database. The total number of pathways retrieved from the KEGG database was 178, of which 57 pathways with relevant information. The remaining pathways were false positive, showed no up- or downregulation or contained too limited information to draw conclusions. The pathways with relevant information that might be important in determining differences in salmonella susceptibility belonged to the following networks: regulation of actin cytoskeleton, apoptosis and regulation of energy metabolism (Te Pas *et al.*, manuscript in preparation). This demonstrates that pathway analysis might be an important tool for the biological interpretation of microarray data and may help in determining the mechanisms that can explain the differences in salmonella susceptibility between chicken lines.

Susceptibility to infectious diseases

To study disease susceptibility in the chicken intestine, disease models affecting the intestine were used. The first model was malabsorption syndrome (MAS). MAS clearly affects the intestine of chickens and therefore is a good model for intestinal health. Also chicken lines with differences in susceptibility to MAS were available, as measured by growth retardation and the severity of the lesions in the intestine (209). However, the aetiology of MAS is still unknown and it is recognised as a multicausal disease involving a combination of pathogens (173). This complicates the interpretation of the results as probably different reactions occur simultaneously during the response to the disease induction and it was unclear which reactions could be expected. *Salmonella enteritidis* was studied as a second model. For salmonella it is important to clearly define disease resistance, as one can measure systemic infection as well as commensal colonization in the gut. Different parameters have been used to define salmonella susceptibility, like differences in lethal doses (28, 62), differences in the bacterial load in liver and spleen (59, 91) and differences in colonization of the gut (45, 91). As colonization by itself is not harmful to the chicken, we concentrated on

resistance to systemic infection as measured by the number of colony forming units in the liver and weight gain depression. The invasion of intestinal epithelial cells is one of the earliest steps in the establishment of systemic infections by enteric pathogens (55). Therefore we decided to investigate gene expression in the intestine immediately after exposure to salmonella. Since salmonella causes serious illness only in young or immuno-compromized chickens, we infected one-day-old broilers. We concentrated on the gene expression responses in the jejunum in order to allow a comparison with the results of the MAS experiments. The jejunum is the most affected part of the GI-tract after MAS induction.

Resistance against salmonella in chicken can be defined in two different ways: resistance against systemic infection and resistance against carrier state. During carrier state the salmonella bacteria remains commensal in the intestine, but the chicken develops no known immune response. Resistance against chicken carrier state is important for food safety reasons. However we were interested in disease susceptibility, therefore we investigated systemic infection and not the carrier state.

In the studies described in this thesis only gene expression in the jejunum has been determined. However, it would also be interesting to investigate gene expression responses to a salmonella infection in other parts of the gastro-intestinal tract. In addition, gene expression in other organs could also be investigated. It is possible that processes in for instance the spleen are also important for salmonella disease susceptibility.

It needs to be established whether selection of chicken lines against a specific pathogen alters susceptibility of these chickens against other pathogens. Chickens resistant to a specific disease might not always be resistant to another disease. So far only a few chicken lines selected for their resistance to a pathogen were tested for susceptibility to another pathogen. For example, an inbred Brown Leghorn line was one of the most robust lines when facing eimeria infections, but was highly susceptible to IBDV infection (29, 30). Age can also complicate genetic susceptibility. The chicken line genetically resistant for IBDV infection at older age, had more-severe B cell depletion after IBDV than a susceptible line when an asymptomatic neonatal infection was induced (152). Also infection dose and route have an effect on genetic disease susceptibility. As an example: three different strains of mice were equally susceptible for a subcutaneously high dose of *Leishmania mexicana* infection, but showed marked differences following an intra-dermal low dose infection (149). In an experiment with three pairs of chicken lines divergently selected for avian leucosis

virus (ALV) the mortality from Marek's disease (MD) was measured (68). In two pairs the ALV resistance correlated with MD resistance, while in the third pair the ALV-resistant strain had a lower MD mortality rate than the ALV-susceptible strain. Hence, the susceptibility spectrum to pathogens other than the one used for selection may vary in different populations. It is expected that the success and consequences of selection for disease resistance vary between different commercial lines of chickens due to different genetic backgrounds (93).

Final conclusions

The scope of this thesis was to identify genes involved in genetic disease resistance and to get insight into the mechanisms that determine differences in susceptibility. Indeed, genes and pathways were identified that varied in regulation between chicken lines with different susceptibilities. However, at this moment only a bit of information is obtained about the underlying mechanisms. These mechanisms should be clarified further before one can identify the key genes, gene sets or expression profiles involved in, or associated with disease susceptibility traits. At this moment no gene or protein sets have been identified that can be used as read-out parameter for intestinal heath in breeding programs or in attempts to optimise intestinal health by nutrition. Nevertheless, the studies described in this thesis have provided evidence that early host responses are important in disease resistance and that studies on disease resistance should consider a broader range of genes, rather than restricting the analysis to genes known to be directly involved in immune defence.

References

1. **Abdrakhmanov, I., D. Lodygin, P. Geroth, H. Arakawa, A. Law, J. Plachy, B. Korn, and J. M. Buerstedde.** 2000. A large database of chicken bursal ESTs as a resource for the analysis of vertebrate gene function. Genome Res. **10:**2062-2069.
2. **Afshari, C. A.** 2002. Perspective: Microarray Technology, Seeing More Than Spots. Endocrinology **143:**1983-1989.
3. **Akira, S., and K. Takeda.** 2004. Toll-like receptor signalling. Nat. Rev. Immunol. **4:**499-511.
4. **Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter.** 2002. Molecular Biology of the Cell, 4 th ed. Garland Science, New York.
5. **Aldridge, P. D., M. A. Gray, B. H. Hirst, and C. M. Khan.** 2005. Who's talking to whom? Epithelial-bacterial pathogen interactions. Mol. Microbiol. **55:**655-663.
6. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.
7. **Amundson, S. A., M. Bittner, P. Meltzer, J. Trent, and A. J. Fornace.** 2001. Physiological function as regulation of large transcriptional programs: the cellular response to genotoxic stress. Comp. Biochem. Physiol. B-Biochem. Mol. Biol. **129:**703-710.
8. **Asheg, A., M. Levkut, V. Revajova, Z. Sevcikova, L. Kolodzieyski, and J. Pistl.** 2002. T lymphocyte subpopulations and B lymphocyte cells in caecum and spleen of chicks infected with Salmonella enteritidis. Acta. Histochem. **104:**435-439.
9. **Athman, R., J. Niewohner, D. Louvard, and S. Robine.** 2002. Epithelial cells: Establishment of primary cultures and immortalization, p. 93-113, Molecular Cellular Microbiology, vol. 31. ACADEMIC PRESS INC, San Diego.
10. **Bacon, L. D., H. D. Hunt, and H. H. Cheng.** 2000. A review of the development of chicken lines to resolve genes determining resistance to diseases. Poult. Sci. **79:**1082-1093.
11. **Baker, P. J.** 2005. Genetic control of the immune response in pathogenesis. J. Periodontol. **76:**2042-2046.
12. **Bao, S., K. W. Beagley, M. P. France, J. Shen, and A. J. Husband.** 2000. Interferon-gamma plays a critical role in intestinal immunity against Salmonella typhimurium infection. Immunology **99:**464-472.
13. **Barrow, P. A., M. B. Huggins, M. A. Lovell, and J. M. Simpson.** 1987. Observations on the pathogenesis of experimental Salmonella typhimurium infection in chickens. Res. Vet. Sci. **42:**194-199.
14. **Barua, A., and Y. Yoshimura.** 2004. Ovarian cell-mediated immune response to Salmonella enteritidis infection in laying hens (Gallus domesticus). Poult. Sci. **83:**997-1002.
15. **Baylis, M., and W. Goldmann.** 2004. The genetics of scrapie in sheep and goats. Curr. Mol. Med. **4:**385-396.
16. **Beal, R. K., C. Powers, P. Wigley, P. A. Barrow, P. Kaiser, and A. L. Smith.** 2005. A strong antigen-specific T-cell response is associated with age and genetically dependent resistance to avian enteric salmonellosis. Infect. Immun. **73:**7509-7516.

17. **Befus, A. D., N. Johnston, G. A. Leslie, and J. Bienenstock.** 1980. Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. J. Immunol. **125:**2626-2632.
18. **Berkes, J., V. K. Viswanathan, S. D. Savkovic, and G. Hecht.** 2003. Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. Gut **52:**439-451.
19. **Berndt, A., and U. Methner.** 2001. Gamma/delta T cell response of chickens after oral administration of attenuated and non-attenuated Salmonella typhimurium strains. Vet. Immunol. Immunopathol. **78:**143-161.
20. **Berti, C., B. Fontanella, R. Ferrentino, and G. Meroni.** 2004. Mig12, a novel Opitz syndrome gene product partner, is expressed in the embryonic ventral midline and co-operates with Mid1 to bundle and stabilize microtubules. BMC Cell. Biol. **5:**9.
21. **Bertilsson, P. M., P. Olsson, and K. E. Magnusson.** 2001. Cytokines influence mRNA expression of cytochrome P450 3A4 and MDRI in intestinal cells. J. Pharm. Sci. **90:**638-646.
22. **Bikle, D. D., S. Munson, N. Morrison, and J. Eisman.** 1993. Zipper protein, a newly described tropomyosin-like protein of the intestinal brush border. J. Biol. Chem. **268:**620-626.
23. **Blackwell, J. M., C. H. Barton, J. K. White, T. I. A. Roach, M. A. Shaw, S. H. Whitehead, B. A. Mock, S. Searle, H. Williams, and A. M. Baker.** 1994. Genetic-Regulation of Leishmanial and Mycobacterial Infections - the Lsh/Ity/Bcg Gene Story Continues. Immunol. Lett. **43:**99-107.
24. **Bliss, T. W., J. E. Dohms, M. G. Emara, and C. L. Keeler, Jr.** 2005. Gene expression profiling of avian macrophage activation. Vet. Immunol. Immunopathol. **105:**289-299.
25. **Boardman, P. E., J. Sanz Ezquerro, I. M. Overton, D. W. Burt, E. Bosch, W. T. Fong, C. Tickle, W. R. A. Brown, S. A. Wilson, and S. J. Hubbard.** 2002. A comprehensive collection of chicken cDNAs. Curr. Biol. **12:**1965-1969.
26. **Bryan, E. D., and M. P. Doyle.** 1995. Health risks and consequences of Salmonella and campylobacter jejuni in raw meat. J. Food Prot. **58:**326-344.
27. **Bumstead, N.** 1998. Genetic resistance to avian viruses. Rev. Sci. Tech. **17:**249-255.
28. **Bumstead, N., and P. Barrow.** 1993. Resistance to Salmonella gallinarum, S. pullorum, and S. enteritidis in inbred lines of chickens. Avian Dis. **37:**189-193.
29. **Bumstead, N., and B. J. Millard.** 1992. Variation in susceptibility of inbred lines of chickens to seven species of Eimeria. Parasitology **104 (Pt 3):**407-413.
30. **Bumstead, N., R. L. Reece, and J. K. Cook.** 1993. Genetic differences in susceptibility of chicken lines to infection with infectious bursal disease virus. Poult. Sci. **72:**403-410.
31. **Burt, D. W.** 2005. Chicken genome: current status and future opportunities. Genome Res. **15:**1692-1698.
32. **Burt, D. W., and P. M. Hocking.** 2002. Mapping quantitative trait loci and identification of genes that control fatness in poultry. Proc. Nutr. Soc. **61:**441-446.

33. **Carre, W., C. Diot, V. Fillon, R. P. Crooijmans, S. Lagarrigue, M. Morrisson, A. Vignal, M. A. Groenen, and M. Douaire.** 2001. Development of 112 unique expressed sequence tags from chicken liver using an arbitrarily primed reverse transcriptase-polymerase chain reaction and single strand conformation gel purification method. Anim. Genet. **32:**289-297.
34. **Cheema, M. A., M. A. Qureshi, and G. B. Havenstein.** 2003. A comparison of the immune response of a 2001 commercial broiler with a 1957 randombred broiler strain when fed representative 1957 and 2001 broiler diets. Poult. Sci. **82:**1519-1529.
35. **Cheeseman, J. H., M. G. Kaiser, C. Ciraci, P. Kaiser, and S. J. Lamont.** 2007. Breed effect on early cytokine mRNA expression in spleen and cecum of chickens with and without Salmonella enteritidis infection. Dev. Comp. Immunol. **31:**52-60.
36. **Cheeseman, J. H., M. G. Kaiser, and S. J. Lamont.** 2004. Genetic line effect on peripheral blood leukocyte cell surface marker expression in chickens. Poult. Sci. **83:**911-916.
37. **Christ, A. D., and R. S. Blumberg.** 1997. The intestinal epithelial cell: Immunological aspects. Springer Seminars in Immunopathology **18:**449-461.
38. **Chu, D. H., C. T. Morita, and A. Weiss.** 1998. The Syk family of protein tyrosine kinases in T-cell activation and development. Immunol. Rev. **165:**167-180.
39. **Cobb, B. S., and S. T. Smale.** 2005. Ikaros-family proteins: in search of molecular functions during lymphocyte development. Curr. Top. Microbiol. Immunol. **290:**29-47.
40. **Collett, A., E. Sims, D. Walker, Y. L. He, J. Ayrton, M. Rowland, and G. Warhurst.** 1996. Comparison of HT29-18-C1 and Caco-2 cell lines as models for studying intestinal paracellular drug absorption. Pharm. Res. **13:**216-221.
41. **Dennis, R., H. M. Zhang, L. D. Bacon, I. Estevez, and H. W. Cheng.** 2004. Behavioral and physiological features of chickens diversely selected for resistance to avian disease. 1. Selected inbred lines differ in behavioral and physical responses to social stress. Poult. Sci. **83:**1489-1496.
42. **Dequeant, M. L., and O. Pourquie.** 2005. Chicken genome: new tools and concepts. Dev. Dyn. **232:**883-886.
43. **Detweiler, C. S., D. B. Cunanan, and S. Falkow.** 2001. Host microarray analysis reveals a role for the Salmonella response regulator phoP in human macrophage cell death. Proc. Natl. Acad. Sci. USA **98:**5850-5855.
44. **Diez-Tascon, C., O. M. Keane, T. Wilson, A. Zadissa, D. L. Hyndman, D. B. Baird, J. C. McEwan, and A. M. Crawford.** 2005. Microarray analysis of selection lines from outbred populations to identify genes involved with nematode parasite resistance in sheep. Physiol. Genomics **21:**59-69.
45. **Duchet-Suchaux, M., F. Mompart, F. Berthelot, C. Beaumont, P. Lechopier, and P. Pardon.** 1997. Differences in frequency, level, and duration of cecal carriage between four outbred chicken lines infected orally with Salmonella enteritidis. Avian Dis. **41:**559-567.

46. **Dudoit, S., Y. H. Yang, M. J. Callow, and T. P. Speed.** 2002. Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. Stat. Sinica **12:**111-139.
47. **Eckmann, L.** 2005. Defence molecules in intestinal innate immunity against bacterial infections. Curr. Opin. Gastroenterol. **21:**147-151.
48. **Eckmann, L., J. R. Smith, M. P. Housley, M. B. Dwinell, and M. F. Kagnoff.** 2000. Analysis by high density cDNA arrays of altered gene expression in human intestinal epithelial cells in response to infection with the invasive enteric bacteria Salmonella. J. Biol. Chem. **275:**14084-14094.
49. **Elo, H. A., M. S. Kulomaa, and P. J. Tuohimaa.** 1979. Avidin induction by tissue injury and inflammation in male and female chickens. Comp. Biochem. Physiol. B **62:**237-240.
50. **Fasenko, G. M., F. E. Robinson, A. I. Whelan, K. M. Kremeniuk, and J. A. Walker.** 2001. Prestorage incubation of long-term stored broiler breeder eggs: 1. Effects on hatchability. Poult. Sci. **80:**1406-1411.
51. **Fearn, R. A., and B. H. Hirst.** 2006. Predicting oral drug absorption and hepatobiliary clearance: Human intestinal and hepatic in vitro cell models. Environment. Toxicol. Pharmacol. **21:**168-178.
52. **Ferro, P. J., C. L. Swaggerty, P. Kaiser, I. Y. Pevzner, and M. H. Kogut.** 2004. Heterophils isolated from chickens resistant to extra-intestinal Salmonella enteritidis infection express higher levels of pro-inflammatory cytokine mRNA following infection than heterophils from susceptible chickens. Epidemiol. Infect. **132:**1029-1037.
53. **Frazier, J. A., and R. L. Reece.** 1990. Infectious stunting syndrome of chickens in Great Britain: intestinal ultrastructural pathology. Avian. Pathol. **19:**459-477
54. **Friedman, A., E. Bar-Shira, and D. Sklan.** 2003. Ontogeny of gut associated immune competence in the chick. World. Poult. Sci. J. **59:**209-219.
55. **Galan, J. E., and R. Curtiss, 3rd.** 1989. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383-6387.
56. **Geiszt, M., and T. L. Leto.** 2004. The Nox family of NAD(P)H oxidases: host defense and beyond. J. Biol. Chem. **279:**51715-51718.
57. **Georges, M.** 2001. Recent progress in livestock genomics and potential impact on breeding programs. Theriogenology **55:**15-21.
58. **Geyra, A., Z. Uni, and D. Sklan.** 2001. Enterocyte dynamics and mucosal development in the posthatch chick. Poult. Sci. **80:**776-782.
59. **Girard-Santosuosso, O., P. Menanteau, M. Duchet-Suchaux, F. Berthelot, F. Mompart, J. Protais, P. Colin, J. F. Guillot, C. Beaumont, and F. Lantier.** 1998. Variability in the resistance of four chicken lines to experimental intravenous infection with Salmonella enteritidis phage type 4. Avian Dis. **42:**462-469.
60. **Gobert, A. P., K. T. Wilson, and C. Martin.** 2005. Cellular responses to attaching and effacing bacteria: activation and implication of the innate immune system. Arch. Immunol. Ther. Exp. (Warsz) **53:**234-244.

61. **Govoni, G., and P. Gros.** 1998. Macrophage NRAMP1 and its role in resistance to microbial infections. Inflamm. Res. **47:**277-284.
62. **Guillot, J. F., C. Beaumont, F. Bellatif, C. Mouline, F. Lantier, P. Colin, and J. Protais.** 1995. Comparison of resistance of various poultry lines to infection by Salmonella enteritidis. Vet. Res. **26:**81-86.
63. **Guiney, D. G., and M. Lesnick.** 2005. Targeting of the actin cytoskeleton during infection by Salmonella strains. Clin. Immunol. **114:**248-255.
64. **Guy, J. S.** 1998. Virus infections of the gastrointestinal tract of poultry. Poult. Sci. **77:**1166-1175.
65. **Hangalapura, B. N., M. G. Kaiser, J. J. van der Poel, H. K. Parmentier, and S. J. Lamont.** 2006. Cold stress equally enhances in vivo pro-inflammatory cytokine gene expression in chicken lines divergently selected for antibody responses. Dev. Comp. Immunol. **30:**503-511.
66. **Hangalapura, B. N., M. G. B. Nieuwland, G. D. Reilingh, J. Buyse, H. Van Den Brand, B. Kemp, and H. K. Parmentier.** 2005. Severe feed restriction enhances innate immunity but suppresses cellular immunity in chicken lines divergently selected for antibody responses. Poult. Sci. **84:**1520-1529.
67. **Harmon, B. G.** 1998. Avian heterophils in inflammation and disease resistance. Poult. Sci. **77:**972-977.
68. **Hartmann, W., D. vd Hagen, and G. Heil.** 1984. Genetic resistance to Leukosis virus infection: effects on mortality and egg production in Leghorn hens. Br. Poult. Sci. **25:**43-52.
69. **Henderson, S. C., D. I. Bounous, and M. D. Lee.** 1999. Early events in the pathogenesis of avian salmonellosis. Infect. Immun. **67:**3580-3586.
70. **Hikima, J., S. Minagawa, I. Hirono, and T. Aoki.** 2001. Molecular cloning, expression and evolution of the Japanese flounder goose-type lysozyme gene, and the lytic activity of its recombinant protein. Biochim. Biophys. Acta **1520:**35-44.
71. **Honjo, K., and Y. Hirota.** 1993. Immunohistochemical investigations of lymphocytes in the lymphoid organs of cyclophosphamide treated chickens. J. Vet. Med. Sci. **55:**895-897.
72. **Hu, J., N. Bumstead, P. Barrow, G. Sebastiani, L. Olien, K. Morgan, and D. Malo.** 1997. Resistance to salmonellosis in the chicken is linked to NRAMP1 and TNC. Genome Res. **7:**693-704.
73. **Hughes, E. A., and J. E. Galan.** 2002. Immune response to Salmonella: location, location, location? Immunity **16:**325-328.
74. **International Chicken Genome Sequencing Consortium.** 2004. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature **432:**695-716.
75. **Ishijima, S. A., Y. X. Zeng, C. Kurashima, M. Utsuyama, T. Shirasawa, K. Sakamoto, and K. Hirokawa.** 1995. Expression of ZAP-70 gene in the developing thymus and various nonlymphoid tissues of embryonic and adult mice. Cell. Immunol. **165:**278-283.
76. **Janeway, C. A., P. Travers, M. Walport, and J. D. Capra.** 1999. immunobiology, the immune system in health and disease. Current biology publications.

77. **Jenner, R. G., and R. A. Young.** 2005. Insights into host responses against pathogens from transcriptional profiling. Nat. Rev. Microbiol. **3:**281-294.
78. **Jeurissen, S. H., E. M. Janse, G. Koch, and G. F. de Boer.** 1988. The monoclonal antibody CVI-ChNL-68.1 recognizes cells of the monocyte-macrophage lineage in chickens. Dev. Comp. Immunol. **12:**855-864.
79. **Jeurissen, S. H. M., L. Vervelde, and E. M. Janse.** 1993. Structure and function of lymphoid tissues of the chicken. Poult sci rev. Oxford, England : Elsevier Science Limited **5:**183-207.
80. **Kaiser, P., T. Y. Poh, L. Rothwell, S. Avery, S. Balu, U. S. Pathania, S. Hughes, M. Goodchild, S. Morrell, M. Watson, N. Bumstead, J. Kaufman, and J. R. Young.** 2005. A genomic analysis of chicken cytokines and chemokines. J. Interferon Cytokine Res. **25:**467-484.
81. **Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley.** 2000. Differential cytokine expression in avian cells in response to invasion by Salmonella typhimurium, Salmonella enteritidis and Salmonella gallinarum. Microbiology **146:**3217-3226.
82. **Kaiser, P., G. Underwood, and F. Davison.** 2003. Differential cytokine responses following Marek's disease virus infection of chickens differing in resistance to Marek's disease. J. Virol. **77:**762-768.
83. **Kanehisa, M., S. Goto, M. Hattori, K. F. Aoki-Kinoshita, M. Itoh, S. Kawashima, T. Katayama, M. Araki, and M. Hirakawa.** 2006. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res. **34:**D354-357.
84. **Karsten, S. L., V. M. D. Van Deerlin, C. Sabatti, L. H. Gill, and D. H. Geschwind.** 2002. An evaluation of tyramide signal amplification and archived fixed and frozen tissue in microarray gene expression analysis. Nucleic Acids Res. **30:**E4.
85. **Keane, O. M., A. Zadissa, T. Wilson, D. L. Hyndman, G. J. Greer, D. B. Baird, A. F. McCulloch, A. M. Crawford, and J. C. McEwan.** 2006. Gene expression profiling of naive sheep genetically resistant and susceptible to gastrointestinal nematodes. BMC Genomics **7:**42.
86. **Kidd, M. T.** 2004. Nutritional modulation of immune function in broilers. Poult. Sci. **83:**650-657.
87. **Klasing, K. C.** 1998. Nutritional modulation of resistance to infectious diseases. Poult. Sci. **77:**1119-1125.
88. **Koenen, M. E., A. G. Boonstra-Blom, and S. H. Jeurissen.** 2002. Immunological differences between layer- and broiler-type chickens. Vet. Immunol. Immunopathol. **89:**47-56.
89. **Kogut, M. H., G. I. Tellez, E. D. McGruder, B. M. Hargis, J. D. Williams, D. E. Corrier, and J. R. DeLoach.** 1994. Heterophils are decisive components in the early responses of chickens to Salmonella enteritidis infections. Microb. Pathog. **16:**141-51.
90. **Kramer, J., M. Malek, and S. J. Lamont.** 2003. Association of twelve candidate gene polymorphisms and response to challenge with Salmonella enteritidis in poultry. Anim. Genet. **34:**339-348.

91. **Kramer, J., A. H. Visscher, J. A. Wagenaar, A. G. Boonstra Blom, and S. H. Jeurissen.** 2001. Characterization of the innate and adaptive immunity to Salmonella enteritidis PT1 infection in four broiler lines. Vet. Immunol. Immunopathol. **79:**219-233.
92. **Kramer, J., A. H. Visscher, J. A. Wagenaar, J. B. Cornelissen, and S. H. Jeurissen.** 2003. Comparison of natural resistance in seven genetic groups of meat-type chicken. Br. Poult. Sci. **44:**577-585.
93. **Kuhnlein, U., S. E. Aggrey, and D. Zadworny.** 2003. Progress and prospects in resistance to disease, p. 283-292. *In* M. W.M. and S. E. Aggrey (ed.), poultry Genetics, Breeding and Biotechnology. CABI publishing.
94. **Kuo, C. T., and J. M. Leiden.** 1999. Transcriptional regulation of T lymphocyte development and function. Annu. Rev. Immunol. **17:**149-187.
95. **Lam Yuk Tseung, S., and P. Gros.** 2003. Genetic control of susceptibility to bacterial infections in mouse models. Cell. Microbiol. **5:**299-313.
96. **Lamont, S. J.** 1998. Impact of genetics on disease resistance. Poult. Sci. **77:**1111-1118.
97. **Lamont, S. J., M. G. Kaiser, and W. Liu.** 2002. Candidate genes for resistance to Salmonella enteritidis colonization in chickens as detected in a novel genetic cross. Vet. Immunol. Immunopathol. **87:**423-428.
98. **Leshchinsky, T. V., and K. C. Klasing.** 2001. Divergence of the inflammatory response in two types of chickens. Dev. Comp. Immunol. **25:**629-638.
99. **Levy, E., Y. Rizwan, L. Thibault, G. Lepage, S. Brunet, L. Bouthillier, and E. Seidman.** 2000. Altered lipid profile, lipoprotein composition, and oxidant and antioxidant status in pediatric Crohn disease. Am. J. Clin. Nutr. **71:**807-815.
100. **Lillehoj, H. S., and K. S. Chung.** 1992. Postnatal development of T-lymphocyte subpopulations in the intestinal intraepithelium and lamina propria in chickens. Vet. Immunol. Immunopathol. **31:**347-360.
101. **Lillehoj, H. S., and J. M. Trout.** 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites. Clin. Microbiol. Rev. **9:**349-360.
102. **Lippi, G., V. Braga, S. Adami, and G. Guidi.** 1998. Modification of serum apolipoprotein A-I, apolipoprotein B and lipoprotein(a) levels after bisphosphonates-induced acute phase response. Clin. Chim. Acta **271:**79-87.
103. **Liu, F. T., R. J. Patterson, and J. L. Wang.** 2002. Intracellular functions of galectins. Biochim. Biophys. Acta **1572:**263-273.
104. **Liu, H. C., H. H. Cheng, V. Tirunagaru, L. Sofer, and J. Burnside.** 2001. A strategy to identify positional candidate genes conferring Marek's disease resistance by integrating DNA microarrays and genetic mapping. Anim. Genet. **32:**351-359.
105. **Liu, M., S. J. Popper, K. H. Rubins, and D. A. Relman.** 2006. Early days: genomics and human responses to infection. Curr. Opin. Microbiol. **9:**312-319.
106. **Liu, W., M. G. Kaiser, and S. J. Lamont.** 2003. Natural resistance-associated macrophage protein 1 gene polymorphisms and response to vaccine against or challenge with Salmonella enteritidis in young chicks. Poult. Sci. **82:**259-266.

107. **Malek, M., J. R. Hasenstein, and S. J. Lamont.** 2004. Analysis of chicken TLR4, CD28, MIF, MD-2, and LITAF genes in a Salmonella enteritidis resource population. Poult. Sci. **83:**544-549.
108. **Malek, M., and S. J. Lamont.** 2003. Association of INOS, TRAIL, TGF-beta2, TGF-beta3, and IgL genes with response to Salmonella enteritidis in poultry. Genet. Sel. Evol. **35 Suppl 1:**S99-111.
109. **Marsland, A. L., E. A. Bachen, S. Cohen, B. Rabin, and S. B. Manuck.** 2002. Stress, immune reactivity and susceptibility to infectious disease. Physiol. Behav. **77:**711-716.
110. **Martin, F., F. Malergue, G. Pitari, J. M. Philippe, S. Philips, C. Chabret, S. Granjeaud, M. G. Mattei, A. J. Mungall, P. Naquet, and F. Galland.** 2001. Vanin genes are clustered (human 6q22-24 and mouse 10A2B1) and encode isoforms of pantetheinase ectoenzymes. Immunogenetics **53:**296-306.
111. **Mast, J., and B. M. Goddeeris.** 1999. Development of immunocompetence of broiler chickens. Vet. Immunol. Immunopathol. **70:**245-256.
112. **Mastroeni, P., J. A. Chabalgoity, S. J. Dunstan, D. J. Maskell, and G. Dougan.** 2001. Salmonella: immune responses and vaccines. Vet. J. **161:**132-164.
113. **Mastroeni, P., and M. Sheppard.** 2004. Salmonella infections in the mouse model: host resistance factors and in vivo dynamics of bacterial spread and distribution in the tissues. Microbes Infect. **6:**398-405.
114. **McCarthy, F. M., A. M. Cooksey, N. Wang, S. M. Bridges, G. T. Pharr, and S. C. Burgess.** 2006. Modeling a whole organ using proteomics: the avian bursa of Fabricius. Proteomics **6:**2759-2771.
115. **McNulty, M. S., and J. B. McFerran.** 1993. The runting stunting syndrome general assessment., p. 519-528. *In* J. B. a. M. M. McFerran (ed.), Virus infections of birds. Elsevier, Amsterdam.
116. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607-625.
117. **Medina, E., and A. Lengeling.** 2005. Genetic regulation of host responses to group A streptococcus in mice. Brief Funct. Genomic Proteomic **4:**248-57.
118. **Meltzer, P. S.** 2001. Spotting the target: microarrays for disease gene discovery. Curr. Opin. Genet. Dev. **11:**258-263.
119. **Min, W., H. S. Lillehoj, S. Kim, J. J. Zhu, H. Beard, N. Alkharouf, and B. F. Matthews.** 2003. Profiling local gene expression changes associated with Eimeria maxima and Eimeria acervulina using cDNA microarray. Appl. Microbiol. Biotechnol. **62:**392-399.
120. **Mittrucker, H. W., and S. H. Kaufmann.** 2000. Immune response to infection with Salmonella typhimurium in mice. J. Leukoc. Biol. **67:**457-463.
121. **Molmenti, E. P., T. Ziambaras, and D. H. Perlmutter.** 1993. Evidence for an acute phase response in human intestinal epithelial cells. J. Biol. Chem. **268:**14116-14124.
122. **Moody, D. E.** 2001. Genomics techniques: an overview of methods for the study of gene expression. J. Anim. Sci. **79:**E128-E135.

123. **Moore, R. J., T. J. Doran, T. G. Wise, S. Riddell, K. Granger, T. M. Crowley, K. A. Jenkins, A. J. Karpala, A. G. D. Bean, and J. W. Lowenthal.** 2005. Chicken functional genomics: an overview. Austr. J. Exp. Agric. **45:**749-756.
124. **Morgan, R. W., L. Sofer, A. S. Anderson, E. L. Bernberg, J. Cui, and J. Burnside.** 2001. Induction of host gene expression following infection of chicken embryo fibroblasts with oncogenic Marek's disease virus. J. Virol. **75:**533-539.
125. **Mueller, A., D. S. Merrell, J. Grimm, and S. Falkow.** 2004. Profiling of microdissected gastric epithelial cells reveals a cell type-specific response to Helicobacter pylori infection. Gastroenterology **127:**1446-1462.
126. **Nacken, W., J. Roth, C. Sorg, and C. Kerkhoff.** 2003. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc. Res. Tech. **60:**569-580.
127. **Nakamura, H., T. Katahira, T. Sato, Y. Watanabe, and J. Funahashi.** 2004. Gain- and loss-of-function in chick embryos by electroporation. Mech. Dev. **121:**1137-1143.
128. **Nakano, T., and T. Graf.** 1991. Goose-type lysozyme gene of the chicken: sequence, genomic organization and expression reveals major differences to chicken-type lysozyme gene. Biochim. Biophys. Acta **1090:**273-276.
129. **Neiman, P. E., A. Ruddell, C. Jasoni, G. Loring, S. J. Thomas, K. A. Brandvold, R. Lee, J. Burnside, and J. Delrow.** 2001. Analysis of gene expression during myc oncogene-induced lymphomagenesis in the bursa of Fabricius. Proc. Natl. Acad. Sci. USA **98:**6378-6383.
130. **Nile, C. J., C. L. Townes, G. Michailidis, B. H. Hirst, and J. Hall.** 2004. Identification of chicken lysozyme g2 and its expression in the intestine. Cell. Mol. Life Sci. **61:**2760-2766.
131. **Nocentini, G., and C. Riccardi.** 2005. GITR: a multifaceted regulator of immunity belonging to the tumor necrosis factor receptor superfamily. Eur. J. Immunol. **35:**1016-1022.
132. **Noy, Y., and D. Sklan.** 1997. Posthatch development in poultry. J. Appl. Poult. Res. **6:**344-354.
133. **Okuducu, A. F., J. C. Hahne, A. Von Deimling, and N. Wernert.** 2005. Laser-assisted microdissection, techniques and applications in pathology (Review). Int. J. Mol. Med. **15:**763-769.
134. **Oswald, I. P.** 2006. Role of intestinal epithelial cells in the innate immune defence of the pig intestine. Vet. Res. **37:**359-368.
135. **Patel, J. C., and J. E. Galan.** 2005. Manipulation of the host actin cytoskeleton by Salmonella--all in the name of entry. Curr. Opin. Microbiol. **8:**10-15.
136. **Peebles, E. D., T. Pansky, S. M. Doyle, C. R. Boyle, T. W. Smith, M. A. Latour, and P. D. Gerard.** 1998. Effects of dietary fat and eggshell cuticle removal on egg water loss and embryo growth in broiler hatching eggs. Poult. Sci. **77:**1522-1530.
137. **Rabsch, W., H. Tschape, and A. J. Baumler.** 2001. Non-typhoidal salmonellosis: emerging problems. Microbes Infect. **3:**237-247.

138. **Rajeevan, M. S., S. D. Vernon, N. Taysavang, and E. R. Unger.** 2001. Validation of array-based gene expression profiles by real-time (kinetic) RT-PCR. J. Mol. Diagn. **3:**26-31.
139. **Rebel, J. M., F. R. Balk, and W. J. Boersma.** 2005. Cytokine responses in broiler lines that differ in susceptibility to malabsorption syndrome. Br. Poult. Sci. **46:**679-686.
140. **Rebel, J. M. J., F. R. M. Balk, J. Post, S. Van Hemert, B. Zekarias, and N. Stockhofe.** 2006. Malabsorption syndrome in broilers. World. Poult. Sci. J. **62:**17-29.
141. **Rebel, J. M. J., J. T. P. van Dam, B. Zekarias, F. R. M. Balk, J. Post, A. F. Minambres, and A. ter Huurne.** 2004. Vitamin and trace mineral content in feed of breeders and their progeny: effects of growth, feed conversion and severity of malabsorption syndrome of broilers. Br. Poult. Sci. **45:**201-209.
142. **Reece, R. L., and J. A. Frazier.** 1990. Infectious stunting syndrome of chickens in Great Britain: field and experimental studies. Avian Pathol. **19:**723-758.
143. **Reece, R. L., P. T. Hooper, S. H. Tate, V. D. Beddome, W. M. Forsyth, P. C. Scott, and D. A. Barr.** 1984. Field, clinical and pathological observations of a runting and stunting syndrome in broilers. Vet. Res. **115:**483-485.
144. **Rehli, M., S. W. Krause, M. Kreutz, and R. Andreesen.** 1995. Carboxypeptidase M is identical to the MAX.1 antigen and its expression is associated with monocyte to macrophage differentiation. J. Biol. Chem. **270:**15644-15649.
145. **Reis, L. H., L. T. Gama, and M. C. Soares.** 1997. Effects of short storage conditions and broiler breeder age on hatchability, hatching time, and chick weights. Poult. Sci. **76:**1459-1466.
146. **Renton, K. W.** 2001. Alteration of drug biotransformation and elimination during infection and inflammation. Pharmacol. Ther. **92:**147-163.
147. **Richter, G., and S. Burdach.** 2004. ICOS: a new costimulatory ligand/receptor pair and its role in T-cell activion. Onkologie **27:**91-95.
148. **Rogojina, A. T., W. E. Orr, B. K. Song, and E. E. Geisert, Jr.** 2003. Comparing the use of Affymetrix to spotted oligonucleotide microarrays using two retinal pigment epithelium cell lines. Mol. Vis. **9:**482-496.
149. **Rosas, L. E., T. Keiser, J. Barbi, A. A. Satoskar, A. Septer, J. Kaczmarek, C. M. Lezama-Davila, and A. R. Satoskar.** 2005. Genetic background influences immune responses and disease outcome of cutaneous L. mexicana infection in mice. Int. Immunol. **17:**1347-1357.
150. **Rosenberger, C. M., A. J. Pollard, and B. B. Finlay.** 2001. Gene array technology to determine host responses to Salmonella. Microbes Infect. **3:**1353-1360.
151. **Roy, M. F., and D. Malo.** 2002. Genetic regulation of host responses to Salmonella infection in mice. Genes Immun. **3:**381-393.
152. **Ruby, T., C. Whittaker, D. R. Withers, M. K. Chelbi-Alix, V. Morin, A. Oudin, J. R. Young, and R. Zoorob.** 2006. Transcriptional profiling reveals a possible role for the timing of the inflammatory response in determining susceptibility to a viral infection. J. Virol. **80:**9207-9216.

153. **Ruchaud, S., N. Korfali, P. Villa, T. J. Kottke, C. Dingwall, S. H. Kaufmann, and W. C. Earnshaw.** 2002. Caspase-6 gene disruption reveals a requirement for lamin A cleavage in apoptotic chromatin condensation. EMBO J. **21:**1967-1977.
154. **Sadeyen, J. R., J. Trotereau, P. Velge, J. Marly, C. Beaumont, P. A. Barrow, N. Bumstead, and A. C. Lalmanach.** 2004. Salmonella carrier state in chicken: comparison of expression of immune response genes between susceptible and resistant animals. Microbes Infect. **6:**1278-1286.
155. **Sambruy, Y., S. Ferruzza, G. Ranaldi, and I. De Angelis.** 2001. Intestinal cell culture models: applications in toxicology and pharmacology. Cell. Biol. Toxicol. **17:**301-317.
156. **Santoro, M. M., G. Gaudino, and P. C. Marchisio.** 2003. The MSP receptor regulates alpha6beta4 and alpha3beta1 integrins via 14-3-3 proteins in keratinocyte migration. Dev. Cell **5:**257-271.
157. **Santos, R. L., R. M. Tsolis, A. J. Baumler, and L. G. Adams.** 2003. Pathogenesis of Salmonella-induced enteritis. Braz. J. Med. Biol. Res. **36:**3-12.
158. **Sasai, K., M. Aita, H. S. Lillehoj, T. Miyamoto, T. Fukata, and E. Baba.** 2000. Dynamics of lymphocyte subpopulation changes in the cecal tonsils of chickens infected with Salmonella enteritidis. Vet. Microbiol. **74:**345-351.
159. **Schalkwijk, J., O. Wiedow, and S. Hirose.** 1999. The trappin gene family: proteins defined by an N-terminal transglutaminase substrate domain and a C-terminal four-disulphide core. Biochem. J. **340:**569-577.
160. **Schat, K. A., and T. J. Myers.** 1991. Avian intestinal immunity. Crit. Rev. Poult. Biol. New York, N.Y. : Elsevier Science Publishing Co**:**19-34.
161. **Schmidt, G., and K. Richter.** 2000. Expression pattern of XIRG, a marker for non-neural ectoderm. Dev. Genes Evol. **210:**575-578.
162. **Schoots, I. G., M. Levi, A. K. van Vliet, A. M. Maas, E. H. Roossink, and T. M. van Gulik.** 2004. Inhibition of coagulation and inflammation by activated protein C or antithrombin reduces intestinal ischemia/reperfusion injury in rats. Crit. Care Med. **32:**1375-1383.
163. **Sheela, R. R., U. Babu, J. Mu, S. Elankumaran, D. A. Bautista, R. B. Raybourne, R. A. Heckert, and W. Song.** 2003. Immune responses against Salmonella enterica serovar enteritidis infection in virally immunosuppressed chickens. Clin. Diagn. Lab. Immunol. **10:**670-679.
164. **Shin, H. H., M. H. Lee, S. G. Kim, Y. H. Lee, B. S. Kwon, and H. S. Choi.** 2002. Recombinant glucocorticoid induced tumor necrosis factor receptor (rGITR) induces NOS in murine macrophage. FEBS Lett. **514:**275-280.
165. **Siegel, P. B., M. Blair, W. B. Gross, B. Meldrum, C. Larsen, K. Boa-Amponsem, and D. A. Emmerson.** 2006. Poult performance as influenced by age of dam, genetic line, and dietary vitamin E. Poult. Sci. **85:**939-942.
166. **Sijben, J. W., K. C. Klasing, J. W. Schrama, H. K. Parmentier, J. J. van der Poel, H. F. Savelkoul, and P. Kaiser.** 2003. Early in vivo cytokine genes expression in chickens after challenge with Salmonella typhimurium lipopolysaccharide and modulation by dietary n--3 polyunsaturated fatty acids. Dev. Comp. Immunol. **27:**611-619.

167. **Smith, E., L. Shi, and G. Smith.** 2002. Expressed sequence tags for the chicken genome from a normalized 10-day-old white leghorn whole-embryo cDNA library. 3. DNA sequence analysis of genetic variation in commercial chicken populations. Genome **45:**261-267.
168. **Smith, M. W., M. A. Mitchell, and M. A. Peacock.** 1990. Effects of genetic selection on growth rate and intestinal structure in the domestic fowl (Gallus domesticus). Comp. Biochem. Physiol. A **97:**57-63.
169. **Smith, P. D., C. Ochsenbauer-Jambor, and L. E. Smythies.** 2005. Intestinal macrophages: unique effector cells of the innate immune system. Immunol. Rev. **206:**149-159.
170. **Song, E., S. K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar, and J. Lieberman.** 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat. Med. **9:**347-351.
171. **Songserm, T., B. Engel, D. J. van Roozelaar, G. L. Kok, A. Pijpers, J. M. A. Pol, and A. A. H. M. ter Huurne.** 2002. Cellular immune response in the small intestine of two broiler chicken lines orally inoculated with malabsorption syndrome homogenates. Vet. Immunol. Immunopathol. **85:**51-62.
172. **Songserm, T., J. M. Pol, D. van Roozelaar, G. L. Kok, F. Wagenaar, and A. A. ter Huurne.** 2000. A comparative study of the pathogenesis of malabsorption syndrome in broilers. Avian Dis. **44:**556-567.
173. **Songserm, T., B. Zekarias, D. J. van Roozelaar, R. S. Kok, J. M. A. Pol, A. Pijpers, and A. ter Huurne.** 2002. Experimental reproduction of malabsorption syndrome with different combinations of reovirus, Escherichia coli, and treated homogenates obtained from broilers. Avian Dis. **46:**87-94.
174. **Stagliano, K. E., E. Carchman, and S. Deb.** 2003. real-time polymerase chain reaction quantitation of relative expression of genes modulated by p53 using SYBR(R) green I. Methods Mol. Biol. **234:**73-92.
175. **Sternberg, E. M.** 2006. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. Nat. Rev. Immunol. **6:**318-328.
176. **Suzuki, S.** 1994. Pathogenicity of Salmonella enteritidis in poultry. Int. J. Food Microbiol. **21:**89-105.
177. **Swaggerty, C. L., P. Kaiser, L. Rothwell, I. Y. Pevzner, and M. H. Kogut.** 2006. Heterophil cytokine mRNA profiles from genetically distinct lines of chickens with differential heterophil-mediated innate immune responses. Avian Pathol. **35:**102-108.
178. **Swaggerty, C. L., M. H. Kogut, P. J. Ferro, L. Rothwell, I. Y. Pevzner, and P. Kaiser.** 2004. Differential cytokine mRNA expression in heterophils isolated from Salmonella-resistant and -susceptible chickens. Immunology **113:**139-148.
179. **Townes, C. L., G. Michailidis, C. J. Nile, and J. Hall.** 2004. Induction of cationic chicken liver-expressed antimicrobial peptide 2 in response to Salmonella enterica infection. Infect. Immun. **72:**6987-6993.
180. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA **98:**5116-5121.

181. **Van den Broeck, W., E. Cox, and B. M. Goddeeris.** 1999. Receptor-dependent immune responses in pigs after oral immunization with F4 fimbriae. Infect. Imm.**67:**520-526.
182. **van der Heide, L., D. Lutticken, and M. Horzinek.** 1981. Isolation of avian reovirus as a possible etiologic agent of osteoporosis ("brittle bone disease"; "femoral head necrosis") in broiler chickens. Avian Dis. **25:**847-856.
183. **van Hemert, S., B. H. Ebbelaar, M. A. Smits, and J. M. J. Rebel.** 2003. Generation of EST and microarray resources for functional genomic studies on chicken intestinal health. Anim. Biotechnol. **14:**133-143.
184. **van Hemert, S., A. J. Hoekman, M. A. Smits, and J. M. J. Rebel.** 2004. Differences in intestinal gene expression profiles in broiler lines varying in susceptibility to malabsorption syndrome. Poult. Sci. **83:**1675-1682.
185. **van Hemert, S., A. J. Hoekman, M. A. Smits, and J. M. J. Rebel.** 2006. Early host gene expression responses to a Salmonella infection in the intestine of chickens with different genetic background examined with cDNA and oligonucleotide microarrays. Comp. Biochem. Physiol. D **1:**292-299.
186. **van Hemert, S., A. J. Hoekman, M. A. Smits, and J. M. J. Rebel.** 2006. Gene expression responses to a Salmonella infection in the chicken intestine differ between lines. Vet. Immunol. Immunopathol. **114:**247-258.
187. **Van Immerseel, F., J. De Buck, I. De Smet, J. Mast, F. Haesebrouck, and R. Ducatelle.** 2002. Dynamics of immune cell infiltration in the caecal lamina propria of chickens after neonatal infection with a Salmonella enteritidis strain. Dev. Comp. Immunol. **26:**355-364.
188. **Velge, P., E. Bottreau, P. Quere, P. Pardon, J. C. Nicolle, M. Morisson, D. Bout, and I. Dimier.** 2002. Establishment and characterization of partially differentiated chicken enterocyte cell clones. Eur. J. Cell. Biol. **81:**203-212.
189. **Vreugdenhil, A. C., A. M. Snoek, J. W. Greve, and W. A. Buurman.** 2000. Lipopolysaccharide-binding protein is vectorially secreted and transported by cultured intestinal epithelial cells and is present in the intestinal mucus of mice. J. Immunol. **165:**4561-4566.
190. **Wallace, J. L.** 2001. Prostaglandin biology in inflammatory bowel disease. Gastroenterol. Clin. North. Am. **30:**971-980.
191. **Wallis, T. S., and E. E. Galyov.** 2000. Molecular basis of Salmonella-induced enteritis. Mol. Microbiol. **36:**997-1005.
192. **Wan, Y., and M. W. Kirschner.** 2001. Identification of multiple CDH1 homologues in vertebrates conferring different substrate specificities. Proc. Natl. Acad. Sci. USA **98:**13066-13071.
193. **Watanabe, C., S. Miura, R. Hokari, K. Teramoto, T. Ogino, S. Komoto, Y. Hara, S. Koseki, Y. Tsuzuki, H. Nagata, D. N. Granger, and H. Ishii.** 2002. Spatial heterogeneity of TNF-alpha-induced T cell migration to colonic mucosa is mediated by MAdCAM-1 and VCAM-1. Am. J. Physiol. Gastrointest. Liver Physiol. **283:**G1379-1387.
194. **Weisel, J. W.** 2005. Fibrinogen and fibrin. Adv. Protein Chem. **70:**247-299.
195. **Wigley, P.** 2004. Genetic resistance to Salmonella infection in domestic animals. Res. Vet. Sci. **76:**165-169.

196. **Withanage, G. S., P. Kaiser, P. Wigley, C. Powers, P. Mastroeni, H. Brooks, P. Barrow, A. Smith, D. Maskell, and I. McConnell.** 2004. Rapid expression of chemokines and proinflammatory cytokines in newly hatched chickens infected with Salmonella enterica serovar typhimurium. Infect. Immun. **72:**2152-2159.

197. **Withanage, G. S., K. Sasai, T. Fukata, T. Miyamoto, E. Baba, and H. S. Lillehoj.** 1998. T lymphocytes, B lymphocytes, and macrophages in the ovaries and oviducts of laying hens experimentally infected with Salmonella enteritidis. Vet. Immunol. Immunopathol. **66:**173-184.

198. **Withanage, G. S., K. Sasai, T. Fukata, T. Miyamoto, H. S. Lillehoj, and E. Baba.** 2003. Increased lymphocyte subpopulations and macrophages in the ovaries and oviducts of laying hens infected with Salmonella enterica serovar Enteritidis. Avian Pathol. **32:**583-590.

199. **Withanage, G. S., P. Wigley, P. Kaiser, P. Mastroeni, H. Brooks, C. Powers, R. Beal, P. Barrow, D. Maskell, and I. McConnell.** 2005. Cytokine and chemokine responses associated with clearance of a primary Salmonella enterica serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. Infect. Immun. **73:**5173-5182.

200. **Wong, G. K., B. Liu, J. Wang, Y. Zhang, X. Yang, Z. Zhang, Q. Meng, J. Zhou, D. Li, J. Zhang, P. Ni, S. Li, L. Ran, H. Li, R. Li, H. Zheng, W. Lin, G. Li, X. Wang, W. Zhao, J. Li, C. Ye, M. Dai, J. Ruan, Y. Zhou, Y. Li, X. He, X. Huang, W. Tong, J. Chen, J. Ye, C. Chen, N. Wei, L. Dong, F. Lan, Y. Sun, Z. Yang, Y. Yu, Y. Huang, D. He, Y. Xi, D. Wei, Q. Qi, W. Li, J. Shi, M. Wang, F. Xie, X. Zhang, P. Wang, Y. Zhao, N. Li, N. Yang, W. Dong, S. Hu, C. Zeng, W. Zheng, B. Hao, L. W. Hillier, S. P. Yang, W. C. Warren, R. K. Wilson, M. Brandstrom, H. Ellegren, R. P. Crooijmans, J. J. van der Poel, H. Bovenhuis, M. A. Groenen, I. Ovcharenko, L. Gordon, L. Stubbs, S. Lucas, T. Glavina, A. Aerts, P. Kaiser, L. Rothwell, J. R. Young, S. Rogers, B. A. Walker, A. van Hateren, J. Kaufman, N. Bumstead, S. J. Lamont, H. Zhou, P. M. Hocking, D. Morrice, D. J. de Koning, A. Law, N. Bartley, D. W. Burt, H. Hunt, H. H. Cheng, U. Gunnarsson, P. Wahlberg, L. Andersson, E. Kindlund, M. T. Tammi, B. Andersson, C. Webber, C. P. Ponting, I. M. Overton, et al.** 2004. A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms. Nature **432:**717-722.

201. **Woo, Y., J. Affourtit, S. Daigle, A. Viale, K. Johnson, J. Naggert, and G. Churchill.** 2004. A comparison of cDNA, oligonucleotide, and Affymetrix GeneChip gene expression microarray platforms. J. Biomol. Tech. **15:**276-284.

202. **Xie, H., G. R. Huff, W. E. Huff, J. M. Balog, P. Holt, and N. C. Rath.** 2002. Identification of ovotransferrin as an acute phase protein in chickens. Poult. Sci. **81:**112-120.

203. **Yang, Y. H., S. Dudoit, P. Luu, D. M. Lin, V. Peng, J. Ngai, and T. P. Speed.** 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. **30:**e15.

204. **Yao, J., J. L. Burton, P. Saama, S. Sipkovsky, and P. M. Coussens.** 2001. Generation of EST and cDNA microarray resources for the study of bovine immunobiology. Acta Vet. Scand. **42:**391-405.
205. **Yin, Z. X., J. G. He, W. X. Deng, and S. M. Chan.** 2003. Molecular cloning, expression of orange-spotted grouper goose-type lysozyme cDNA, and lytic activity of its recombinant protein. Dis. Aquat. Organ. **55:**117-123.
206. **Yonash, N., L. D. Bacon, R. L. Witter, and H. H. Cheng.** 1999. High resolution mapping and identification of new quantitative trait loci (QTL) affecting susceptibility to Marek's disease. Anim. Genet. **30:**126-135.
207. **Yoshida, A., M. T. Minowa, S. Takamatsu, T. Hara, S. Oguri, H. Ikenaga, and M. Takeuchi.** 1999. Tissue specific expression and chromosomal mapping of a human UDP-N-acetylglucosamine: alpha1,3-d-mannoside beta1, 4-N-acetylglucosaminyltransferase. Glycobiology **9:**303-310.
208. **You, M., P. T. Ku, R. Hrdlickova, and H. R. Bose, Jr.** 1997. ch-IAP1, a member of the inhibitor-of-apoptosis protein family, is a mediator of the antiapoptotic activity of the v-Rel oncoprotein. Mol. Cell. Biol. **17:**7328-7341.
209. **Zekarias, B., T. Songserm, J. Post, G. L. Kok, and J. M. Pol.** 2002. Development of organs and intestinal mucosa leukocytes in four broiler lines that differ in susceptibility to malabsorption syndrome. Poult Sci **81:**1283-1288.
210. **Zekarias, B., N. Stockhofe-Zurwieden, J. Post, F. Balk, C. van Reenen, E. Gruys, and J. M. Rebel.** 2005. The pathogenesis of and susceptibility to malabsorption syndrome in broilers is associated with heterophil influx into the intestinal mucosa and epithelial apoptosis. Avian Pathol. **34:**402-407.
211. **Zekarias, B., A. Ter Huurne, W. J. M. Landman, J. M. J. Rebel, J. M. A. Pol, and E. Gruys.** 2002. Immunological basis of differences in disease resistance in the chicken. Vet. Res. **33:**109-125.
212. **Zeng, H., A. Q. Carlson, Y. Guo, Y. Yu, L. S. Collier Hyams, J. L. Madara, A. T. Gewirtz, and A. S. Neish.** 2003. Flagellin is the major proinflammatory determinant of enteropathogenic Salmonella. J. Immunol. **171:**3668-3674.

Summary

The gastrointestinal tract is a portal of entry for many pathogens. In young chickens infection of the gastrointestinal tract often occurs. Due to restrictions in the use of antibiotics and the limited availability of effective vaccines, it is relevant to increase the disease resistance of chickens. Environmental conditions play a role in disease resistance, but resistance is for a large part genetically determined. Genetic disease resistance can be studied in different ways, *e.g.* mapping of quantitative trait loci, association studies or by gene expression profiling. Induction of a disease results in changes in the gene expression levels within the host. These changes can differ between susceptible and resistant animals. Differences in gene expression may also occur under non-challenge conditions. The scope of the experiments described in this thesis was to identify genes involved in genetic disease resistance and to get insight into the mechanisms determining differences in disease susceptibility traits. Therefore gene expression profiles were measured with microarrays, in intestinal tissues from chickens that differ in their susceptibility to intestinal infectious diseases. Microarray hybridisations can be performed to study the gene expression levels of thousands of genes simultaneously.

At the moment these research activities started, there was no chicken microarray commercially available. Therefore at first a normalized chicken intestine specific cDNA library was generated (**chapter 2**). This cDNA library was constructed from RNA originating from the jejunum of young chicks and subtracted with RNA derived from chicken breast muscle tissue. Eighty-three randomly chosen clones were sequenced to check the diversity of the library, and 71 different sequences were found. About 10% of the sequences were not present in the chicken DNA databases of that moment. Only 11% of the sequences was found to be expressed in various organs or tissues, including muscle. Intestine specific expression was found for 45% of the sequences whereas for 28% of the sequences expression in the intestine was not described before. More than 3000 clones from this cDNA library were printed on a microarray. This microarray tool was tested by comparing gene expression profiles of intestinal tissue derived from malabsorption syndrome (MAS)-induced and control chickens. Differentially expressed genes could be detected.

The microarray described in chapter 2 was used to investigate gene expression profiles in the intestine of MAS -induced and control chickens (**chapter 3**). Gene expression was examined in the jejunum from 8 hours till 11 days post infection. Two different broiler lines were used that differed in susceptibility to MAS. No significant gene expression differences were found between the two broiler lines under control

conditions at early timepoints, only at day 11 post infection genes were identified differing more than fourfold under control conditions. However, under MAS induced conditions gene expression differences between the two broiler lines were found early after induction. For one set of genes this was due to up- or down-regulation in response to MAS induction only in the most susceptible chicken line. Another set of genes showed a prolonged up- or down-regulation in the susceptible chicken line compared to the resistant line. In the most susceptible line more genes were up- or down-regulated in response to the MAS induction than in the more resistant chicken line. The induced genes included immune related genes, genes associated with feed absorption and genes with unknown functions.

To investigate whether common genes are involved in disease susceptibility a new experiment was performed with another disease model, salmonellosis (**chapter 4**). In this experiment one-day-old chickens were orally inoculated with *Salmonella enteritidis* and gene expression in the jejunum was investigated 1 day till 21 days post infection in two different chicken lines. The two chicken lines differed in the severity of the systemic infection, as measured by the amount of colony forming units in the liver and their growth retardations. Differences in gene expression levels between two broiler lines were detected under control as well as under infected conditions. At day 1 post infection the highest number of genes was identified with expression differences between the infected animals of the two chicken lines. Under control conditions the highest number of gene expression differences between the two chicken lines were observed at days 7 and 9 pi. The gene expression data indicated that the two chicken lines differed from each other after the salmonella infection with regard to their T-cell development, T-cell activation or T-cell responses. The gene expression data also pointed towards a difference in macrophage activation between the two broiler lines.

It was expected that additional genes and pathways involved in salmonella susceptibility could be identified when whole genome oligonucleotide-based microarrays were used instead of "tissue-specific" microarrays. Therefore the RNA samples of 1 day post infection derived from the salmonella infection experiment described in chapter 4 were analysed on a whole genome microarray (**chapter 5**). The results were compared with the data obtained with the homemade cDNA microarray. Five genes were found to be at least twofold upregulated in both lines with the cDNA microarray and four of these genes were also found to be upregulated with the oligonucleotide microarray. About half of the 71 randomly chosen sequences of the cDNA microarray showed similar expression data on the oligonucleotide microarray,

thus different microarray platforms do not always give the same results. With the oligonucleotide microarray genes were identified which were at least twofold upregulated in response to a salmonella infection in both chicken lines. These genes encoded for proteins with functions in the innate immune system or in wound healing. In the most susceptible chicken line genes involved in inflammation were highly upregulated (more than tenfold). Genes with unknown functions were also upregulated in this chicken line. In the more resistant chicken line genes involved in acute phase response, the fibrinogen system, actin polymerisation, and several genes with unknown functions, were highly upregulated.

The latter gene expression data suggested that innate immunity, inflammation and T-cell responses are important processes in the chicken intestine in response to a salmonella infection.. To further validate the gene expression data with more physiological and immunological data a second experiment with salmonella was performed (**chapter 6)**. Again one-day-old broilers from two different chicken lines were orally inoculated with *Salmonella enteritidis*. The two broiler lines differed in the amount of bacteria in the liver and the reduction in body weight gain. The gene expression data 1 day post infection partly confirmed the data obtained in the first salmonella experiment. Again we observed that the two chicken lines had more gene expression differences than common genes expression responses to the salmonella infection. Also macrophage activation seemed to differ between the two chicken lines based on gene expression differences. Immunohistochemistry showed that in the susceptible chicken line the number of macrophages per mm^2 intestinal tissue decreased after the salmonella infection, which was not the case for the resistant chicken line. In the resistant chicken line the number of $CD4^+$ T-cells decreased at 1 and 5 days post infection, which was not the case in the susceptible chicken line. In both chicken lines the number of $CD8^+$ T-cells increased after the infection, but this increase was faster after infection in the susceptible chicken line.

A discussion of the main findings of the preceding chapters and some of the final conclusions are given in **chapter** 7. The first main finding was that a number of genes has been identified that are differentially induced between chicken lines after challenge. Secondly it was found that most changes in gene expression profiles in response to an infection were found early after the infection. The third finding was that the genetic background of chickens largely influenced gene expression responses to an infection, as more differences between the chicken lines were found than gene expression responses in common in response to an infection. For all these findings the

use of microarrays and *in vivo* experiments were important methodological aspects. To unravel the physiological role in disease susceptibility of differentially expressed genes further functional validation should be performed. Nevertheless new insights into genes involved in susceptibility to infectious diseases are obtained in the studies described in this thesis.

Nederlandse samenvatting

Via het maag-darmstelsel komen veel pathogenen het lichaam binnen. In jonge kippen komen veel infecties in het maag-darmstelsel voor. Om infecties te verminderen kunnen antibiotica en vaccins ingezet worden. Door EU wetgeving is het gebruik van antibiotica beperkt en effectieve vaccines zijn beperkt beschikbaar, daarom is het belangrijk om de ziekteresistentie van kippen te verbeteren. Ziekteresistentie kan beïnvloed worden door omgevingsfactoren, maar kan ook voor een groot deel genetisch bepaald zijn. Genetische ziektegevoeligheid kan op verschillende manieren worden bestudeerd, bijvoorbeeld door gen expressie profielen te bekijken. Het introduceren van een ziekte resulteert in veranderingen in gen expressie niveaus in de gastheer. Deze veranderingen kunnen verschillen tussen gevoelige en resistente dieren veroorzaken. Verschillen in gen expressie in de gastheer kunnen voorkomen onder controle condities en tijdens ziekte. Het doel van de experimenten die in dit proefschrift zijn beschreven is om genen te identificeren die betrokken zijn bij genetische ziektegevoeligheid en om inzicht te verkrijgen in de mechanismen die betrokken zijn bij verschillen in ziektegevoeligheid. Hiervoor zijn gen expressie profielen gemeten met microarrays, in darmweefsel van kippen met verschillende gevoeligheid voor infectieziekten in de darm. Microarray hybridisaties worden uitgevoerd om de gen expressie niveaus van duizenden genen tegelijkertijd te bestuderen.

Tijdens het onderzoek dat in dit proefschrift beschreven staat, is gebruik gemaakt van twee verschillende ziektemodellen, malabsorptie syndroom (MAS) en salmonella infectie. MAS is een aandoening bij jonge vleeskuikens die wordt gekarakteriseerd door groeivertraging en een ontsteking van de darm. Het is een multifactoriële aandoening waarbij verschillende virussen en bacteriën een rol spelen. Salmonella kan voedselvergiftiging bij de mens veroorzaken, doordat de mens bijvoorbeeld salmonella besmette eieren of kippenvlees eet. Ook in jonge kippen kan salmonella ernstige ziekten veroorzaken met diarree en uitdrogingsverschijnselen.

Op het moment dat de onderzoeksactiviteiten startten die beschreven staan in dit proefschrift was er geen commercieel verkrijgbare microarray. Daarom is eerst een genormaliseerde kippendarm specifieke cDNA bank gemaakt (**hoofdstuk 2**). Deze cDNA bank is gemaakt van RNA uit het jejunum van jonge kippen en gesubtraheerd met RNA uit de borstspier van kippen. Van 83 willekeurig gekozen klonen is de sequentie bepaald om te diversiteit van de bank te testen, en 71 verschillende sequenties werden gevonden. Ongeveer 10% van de sequenties was niet aanwezig in de kippen DNA databases die op dat moment beschikbaar waren. Slechts 11% van de gevonden sequenties komt tot expressie in verschillende organen en weefsels,

waaronder spierweefsel. Darm specifieke expressie is gevonden voor 45% van de sequenties, terwijl voor 28% van de sequenties expressie in de darm niet eerder was beschreven. Meer dan 3000 klonen van deze cDNA bank werden geprint op een microarray. Deze microarray is getest door gene expressie profielen te vergelijken van darmmateriaal van malabsorptie syndroom geïnduceerde kippen en controle kippen. Er zijn genen gevonden die verschillend tot expressie kwamen in de darm door inductie van MAS.

De microarray die in hoofdstuk 2 is beschreven, is gebruikt om gen expressie profielen te bestuderen in de darmen van MAS geïnduceerde kippen en controle kippen (**hoofdstuk 3**). Gen expressie is bestudeerd in het jejunum acht uur tot 11 dagen na infectie. Twee verschillende kippenlijnen zijn gebruikt die verschillen in gevoeligheid voor MAS. Er werden geen significante verschillen in gen expressie gevonden tussen de twee kippenlijnen onder controle condities op vroege tijdstippen, alleen op 11 dagen na infectie zijn genen geïdentificeerd die meer dan viervoudig verschillen in expressieniveau onder controle condities. Echter, onder MAS geïnduceerde condities zijn wel vroeg na inductie gen expressie verschillen gevonden tussen de twee kippenlijnen. Voor één set genen kwam dit doordat ze omhoog of omlaag gereguleerd werden als reactie op de MAS inductie in de meest gevoelige kippenlijn en niet in de meer resistente kippenlijn. Een andere set van genen had een langere regulatie in de tijd na MAS inductie in de gevoelige kippenlijn vergeleken met de resistente kippenlijn. In de meest gevoelige kippenlijn werden meer genen gereguleerd als reactie op de MAS inductie dan in de meer resistente kippenlijn. Onder de gereguleerde genen zijn immuun gerelateerde genen, genen die geassocieerd zijn met voedselabsorptie en genen met onbekende functies.

Om te bestuderen of er generieke genen betrokken zijn bij ziektegevoeligheid is een nieuw experiment uitgevoerd met een ander ziektemodel, salmonella (**hoofdstuk 4**). In dit experiment zijn één dag oude kippen oraal geïnoculeerd met *Salmonella enteritidis* en gen expressie in het jejunum is bestudeerd van 1 dag tot 21 dagen na infectie in twee verschillende kippenlijnen. De twee kippenlijnen verschilden in de ernst van de systemische infectie, gemeten aan het aantal salmonella bacteriën in de lever en de groei vertraging. Verschillen in gen expressie niveaus tussen de twee kippen lijnen zijn zowel onder controle condities gevonden als onder geïnfecteerde condities. Op dag 1 na infectie zijn de meeste genen gevonden met expressie verschillen tussen de geïnfecteerde dieren van de twee kippenlijnen. Onder controle condities werden de meeste verschillen in genexpressie tussen de twee kippenlijnen gevonden op dag 7 en 9 na infectie. De genexpressie data suggereerden dat de

verschillen na de salmonella infectie tussen de twee kippenlijnen betrekking hebben op T-cel ontwikkeling of T-cel activatie. De gen expressie data gaven ook indicaties voor verschillen in macrofaag activatie tussen de twee kippenlijnen.

Er werd verwacht dat extra genen en biologische mechanismen gevonden konden worden die betrokken zijn bij salmonella gevoeligheid wanneer er hele genoom microarrays werden gebruikt in plaats van weefsel specifieke microarrays. Daarom werd RNA van dag 1 na infectie afkomstig van de salmonella proef die beschreven is in hoofdstuk 4 geanalyseerd op een hele genoom microarray (**hoofdstuk 5**). De resultaten zijn vergeleken met de data die verkregen zijn met de zelfgemaakte weefsel-specifieke cDNA microarray. Er zijn vijf genen gevonden met de cDNA microarray die ten minste tweevoudig omhoog gereguleerd werden in beide kippenlijnen na salmonella infectie en vier van deze genen werden ook gevonden met behulp van de hele genoom microarray. Ongeveer de helft van 71 willekeurig gekozen sequenties van de cDNA microarray gaf na analyse vergelijkbare expressie data op de hele genoom microarray, dus verschillende microarray platformen geven niet altijd dezelfde resultaten. Met de hele genoom microarray zijn genen geïdentificeerd die tenminste tweevoudig omhoog gereguleerd worden als reactie op de salmonella infectie in beide kippenlijnen. Deze genen codeerden voor eiwitten met functies in het aangeboren immuun systeem of in wond genezing. In de meest gevoelige kippenlijn werden genen die betrokken zijn bij ontstekingsreacties meer dan tienmaal opgereguleerd. Ook genen met onbekende functies werden opgereguleerd in deze kippenlijn. In de meer resistente kippen lijnen werden genen meer dan tienmaal opgereguleerd die betrokken zijn bij de acute fase respons, het fibrinogeen systeem en actine polymerisatie. Ook werden verschillende genen met onbekende functies opgereguleerd.

De gen expressie data van het salmonella experiment suggereerde dat het aangeboren immuunsysteem, ontsteking en T-cel reacties belangrijke processen in de kippendarm zijn als reactie op een salmonella infectie. Om de gen expressie data verder te valideren met meer fysiologische en immunologische data is een tweede experiment met een salmonella infectie in jonge kippen uitgevoerd. Opnieuw werden 1 dag oude kuikens van twee verschillende kippenlijnen oraal geïnoculeerd met *Salmonella enteritidis*. De twee vleeskuiken lijnen verschilden in de hoeveelheid salmonella bacteriën in de lever en in de afname van de gewichtsgroei. De gen expressie data van dag 1 na infectie bevestigde gedeeltelijk de data die in het eerste salmonella experiment verkregen zijn. Opnieuw zagen we dat de twee kippenlijnen meer expressie verschillen hadden dan dat gemeenschappelijk gen expressie reacties op de salmonella infectie waren. Ook de macrofaag activatie leek te verschillen tussen de

twee kippenlijnen, gebaseerd op de gen expressie verschillen. Immunohistochemie liet zien dat in de meest gevoelige kippenlijn het aantal macrofagen per vierkante mm darmweefsel afnam na de salmonella infectie. Dit gebeurde niet in de resistente kippenlijn. In de resistente kippenlijn verminderde het aantal $CD4^+$ T-cellen op dag 1 en 5 na infectie en dat gebeurde niet in de gevoelige kippenlijn. In beide kippenlijnen nam het aantal $CD8^+$ T-cellen toe na de infectie, maar deze toename was sneller in de tijd na de infectie in de gevoelige kippenlijn.

Een discussie van de belangrijkste bevindingen van de voorgaande hoofdstukken en een aantal van de laatste conclusies wordt gegeven in **hoofdstuk 7**. De eerste belangrijke bevinding was dat er een aantal genen zijn geïdentificeerd die verschillend worden geïnduceerd tussen kippenlijnen na een infectie. Als tweede conclusie is gevonden dat de meeste veranderingen in de gen expressie profielen in reactie op een infectie vroeg na de infectie worden gevonden. De derde bevinding was dat de genetische achtergrond van kippen een grote invloed heeft op gen expressie reacties op een infectie, omdat we meer verschillen in genexpressie profielen in de darm tussen de kippenlijnen vonden dan gelijke gen expressie reacties na infectie. Voor al deze bevindingen waren het gebruik van microarrays en *in vivo* experimenten belangrijke methodische aspecten. Om de fysiologische rol in ziektegevoeligheid te ontrafelen van de genen die verschillend tot expressie komen, moet verdere functionele validatie worden gedaan. Desalniettemin zijn er tijdens de studies die in deze thesis beschreven staan nieuwe inzichten verkregen in ziektegevoeligheid voor infectieziekten in de kippendarm.

Curriculum vitae

Op 16 februari 1979 werd ik, Saskia van Hemert, geboren in Harderwijk. In 1997 heb ik mijn VWO-diploma gehaald aan O.S.G. de Rietlanden te Lelystad. In hetzelfde jaar ben ik begonnen met de studie Scheikunde aan de Vrije Universiteit in Amsterdam. Voor mijn hoofdvak Biochemie en Moleculaire Biologie heb ik zes maanden stage gelopen bij de afdeling Biochemie en Moleculaire Biologie van de VU. Voor mijn bijvak medische microbiologie heb ik vier maanden stage gelopen bij het ID-Lelystad. In november 2001 ben ik afgestudeerd en vanaf december 2001 ben ik als AIO werkzaam geweest bij Universiteit Wageningen, departement dierwetenschappen en was ik gedetacheerd bij de divisie Dier en Omgeving bij ID-Lelystad (nu Animal Sciences Group van Wageningen UR). Hier heb ik gewerkt aan het onderzoek dat is beschreven in dit proefschrift.

Publications

Van Hemert S, Hoekman AJW, Smits MA, Rebel JMJ. Immunological and gene expression responses to a Salmonella infection in the chicken intestine. Vet. Res. 2007, 38:51-63.

Rebel JMJ, Van Hemert S, Hoekman AJW, Balk FR, Bakker D, Smits MA. Mother diet influence gene expression in the intesine of the offspring. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 2006, 145:502-508

Van Hemert S, Hoekman AJW, Smits MA, Rebel JMJ. Altered early gene expression responses to a Salmonella infection in the intestine of chickens with different genetic background as examined with cDNA and oligonucleotide microarrays. Comp. Biochem. Physiol. D. 2006, 1:292-299.

Van Hemert S, Hoekman AJW, Smits MA, Rebel JMJ. Gene expresssion responses to a Salmonella infection in the chicken intestine differ between lines. Vet. Immunol. Immunopathol. 2006, 114:247-258.

Rebel JMJ, Balk FRM, Post J, Van Hemert S, Zekarias B, Stockhofe N. Malabsorption syndrome in broilers. Worlds Poult. Sci. J. 2006, 62:17-31.

Van Hemert S, Hoekman AJW, Smits MA, Rebel JMJ. Differences in intestinal gene expression profiles in broiler lines varying in susceptibility to malabsorption syndrome. Poult. Sci. 2004, 83:1675-1682.

Van Hemert S, Ebbelaar BH, Smits MA, Rebel JMJ. Generation of EST and microarray resources for functional genomic studies on chicken intestinal health. Anim. Biotechnol. 2003, 14:133-143.

Eppens NA, Faber AW, Rondaij M, Jahangir RS, van Hemert S, Vos JC, Venema J, Raue HA. Deletions in the S1 domain of Rrp5p cause processing at a novel site in ITS1 of yeast pre-rRNA that depends on Rex4p. Nucl. Acids Res. 2002, 30:4222-4231.

Dankwoord

Mijn proefschrift is af en ligt voor jullie. Hieronder wil ik graag een aantal mensen bedanken die aan mijn boekje hebben bijgedragen of op een andere manier belangrijk voor mij zijn geweest de afgelopen periode.

Annemarie, als degene die het onderzoeksvoorstel geschreven heeft en als directe begeleidster ben je erg nauw bij mijn project betrokken geweest. Ik geloof niet dat ik het veel beter had kunnen treffen met mijn begeleidster, je was eigenlijk altijd beschikbaar als ik je nodig had, zelfs toen je met zwangerschapsverlof was. Je enthousiasme, steun en vertrouwen zijn erg belangrijk geweest, bedankt hiervoor!

Mari, tijdens de projectbesprekingen was je altijd erg meedenkend. Jij was degene die me vooral stimuleerde om snel mijn resultaten op te schrijven.

Martien, vanuit Wageningen was je wat meer van een afstand betrokken bij mijn project, maar toch was het voor jou geen probleem om zo nu en dan naar Lelystad te reizen voor overleg. Ook heb je mij de nodige dingen van de bio-informatica laten zien.

Het is onmogelijk voor mij om alle collega's waar ik de afgelopen vijf jaar mee heb samengewerkt te noemen. Mijn promotieonderzoek is gestart bij het ID-Lelystad bij het cluster dierfysiologie en gezondheid binnen de divisie dier en omgeving. De afronding van mijn proefschrift is gebeurd bij de Animal Sciences Group van Wageningen UR bij het cluster infectiebiologie bacteriologie binnen de divisie infectieziekten. Tussendoor zijn mijn werkzaamheden ook nog gevallen binnen het cluster dierfysiologie en genomics en de divisie veehouderij. Toch ga ik een poging doen om een aantal mensen bij naam te noemen.

Bastiaan, door reorganisatie perikelen is jouw bijdrage aan het project wat korter geweest dan in eerste instantie de bedoeling was, maar mede door jouw array ervaring heeft het project wel een vliegende start gemaakt. Arjan, jij hebt me ontzettend veel geholpen met de labwerkzaamheden en vooral ook met de dierproeven. Frank, Stéphanie, Pieter, Pim, Ina, Christine, Marco en Luc bedankt voor de hulp in de beginperiode met het opzetten van de array hybridisaties en de analyses. Dirk, jouw werk als stagiair is heel waardevol geweest en heeft uiteindelijk aanleiding gegeven tot een artikel. Willem en Gabriele, het was gezellig om met jullie op een kamer te zitten

en regelmatig onze resultaten te bediscussiëren. De overige, huidige, bewoners van vleugel 15; Marinus, Jan, Agnes, Marcel, Jaap, Conny, Herma, José, Helmi, Astrid, Henk, Hilde, Jelle, Michiel, Leo, Ditta en Hélène, bedankt voor de gezelligheid in de pauzes en de oprechte belangstelling. Dit geldt evenzeer voor alle mensen die inmiddels van vleugel 15 (of vleugel 19, ik ben heel wat keer heen en weer gelopen, gefietst en gestept) verhuisd zijn. Met name wil ik daarbij nog de “kippencollega’s” noemen: Bereket, Francis en Jacob, jullie zijn inmiddels ergens anders werkzaam, maar in het begin van mijn project heb ik veel aan de samenwerking met julie gehad.

Astrid, jij ontzettend bedankt voor de mooie kaft. Het is maar goed dat ik ook creatieve personen ken, mezelf was het nooit zo gelukt.

Voor mijn werk was er ook van familie en vrienden veel belangstelling, ook al begrepen de meeste er niet zoveel van. Vrienden waren altijd in om een potje te tennissen, voor een spelletjesavond, een “biertje doen” in Amsterdam, een etentje en natuurlijk de kerstdiners. Astrid en Kim, ik weet zeker dat er van jullie ook een boekje komt.

Marjolein en Jolanda, met drie meiden thuis is gewoon heel erg gezellig, ik ben blij dat we zo’n goede band hebben. En jullie durven het nog steeds aan om samen met mij op de tennisbaan te gaan staan.

Pap en mam, jullie hebben mij altijd gestimuleerd en de mogelijkheden geboden om alles uit mezelf te halen. Het is fijn om te weten dat jullie altijd met me meeleven en me altijd willen helpen.

Lieve Lisanne, je hebt zo’n leuke lach en je kan zo gezellig kletsen. Lieve Jasper, je bent nog zo klein en toch word je ook alweer zo groot. Jullie komst is de mooiste onderbreking van mijn werk geweest die ik had kunnen bedenken. Het is mooi om jullie steeds meer te zien ontdekken van de wereld.

Lieve Marcel, ik ben blij ik je mijn man mag noemen. Jouw rust, relativeringsvermogen en steun zijn ontzettend belangrijk voor me en ik ben heel blij dat wij ons leven samen met de kinderen kunnen delen.

Saskia

PhD Education plan

Training and Supervision Plan			**Graduate School WIAS**
Name PhD student	Saskia van Hemert		
Project title	Toolbox preparation and utility concerning chicken intestinal health		
Group	Animal Breeding and Genetics		
Daily supervisor(s)	Annemarie Rebel		
Supervisor(s)	Mari Smits, Martien Groenen		
Project term	from 01-12-2001	until 23-09-2006	
Submitted	1-9-2006	first plan / midterm / **certificate**	

EDUCATION AND TRAINING (minimum 30, maximum 60 credits)		
The Basic Package (minimum 3 credits)	year	credits*
WIAS Introduction Course (mandatory)	2003	
WIAS Course on philosophy of science and/or ethics (mandatory)	2002	
Subtotal Basic Package		**3**
Scientific Exposure (conferences, seminars and presentations, minimum 8 credits)	year	
International conferences *(minimum 3 credits)*		
ESF Functional Genomics and Diseases conference, Prague (Czech republic) 14-17 May	2003	
3rd European Poultry Genetics Symposium, Wageningen 17-19 September	2003	
1st International symposium on networks in bioinformatics, Amsterdam 22-23 March	2004	
Genetics on animal health, Ames (Iowa, VS) 13-15 juli	2005	
Seminars and workshops		
Pluimveeonderzoekdag, Lelystad 15 March	2002	
Veterinary Science Day, Garderen, 14 November 2002 and 12 November 2003	2002/03	
Immunologic and genetic aspects of resistance to Salmonella, Lelystad 19 March	2003	
WIAS Science Day, Wageningen, 27 March 2003, 25 March 2004, 17 February 2005	2003-05	
Decisions in genomics, Utrecht 20 January	2004	
Antigen presentation at the mucosal surface, Rotterdam 3-5 March	2004	
Integrating proteomics in biology, Utrecht 6 April	2004	
PhD Retreat Unity in Diversity, Nijmegen, 13-14 May	2004	
Selection of chickens; an approach to unravel specific and innate immune competence, Wageningen, March 9	2005	
Presentations *(minimum 4 original presentations of which at least 1 oral, 1 credit each)*		
Human Centre of Nutrigenomics, intestinal function, Wageningen 18 September (oral)	2002	
ESF Functional Genomics and Diseases conference, Prague (Czech republic) 14-17 May (poster)	2003	
Nederlandse darmendag, Wageningen 31 October (oral)	2003	
3rd European Poultry Genetics Symposium, Wageningen 17-19 September (poster)	2003	
PhD Retreat Unity in Diversity, Nijmegen, 13-14 May, (oral)	2004	
WIAS Science Day, Wageningen, 17 February (oral + poster)	2005	
Genetics on animal health, Ames (Iowa, VS) 13-16 July (oral + poster)	2005	
Subtotal International Exposure		**16**

In-Depth Studies (minimum 6 credits)	year	credits*
Disciplinary and interdisciplinary courses		
Bioinformatics, Wageningen, Laboratory of biochemistry, 8 - 19 April	2002	
Immunology, Eijkman graduate school, 6 -10 January	2003	
Infectious biology, Eijkman graduate school, 13 -16 January	2003	
Ecophysiology of the Gastro-Intestinal tract, VLAG, 28 February - 3 March	2005	
Advanced statistics courses		
Statistics for researchers, Lelystad, June -July	2002	
Subtotal In-Depth Studies		**10**
Professional Skills Support Courses (minimum 3 credits)	year	
Scientific Writing (by Linda Mcfee, at ID-Lelystad)	2002	
Research management, NIBI, 9 -11 December	2003	
Subtotal Professional Skills Support Courses		**5**
Didactic Skills Training (optional)	year	
Supervising minor MSc thesis	2004	
Subtotal Didactic Skills Training		**2**
Management Skills Training (optional)	year	
General member of the WAPS Council	2004	
Subtotal Management Skills Training		**2**
Education and Training Total (minimum 30, maximum 60 credits)		**38**
* one ECTS (European Credit Transfer System) credit equals a study load of approximately 28 hours		

Notes

This study was conducted at the Animal Sciences Group (ASG) in Lelystad

The printing of this thesis was financially supported by:

Wageningen University, Wageningen

Animal Sciences Group, Lelystad

Cover design:
Astrid Sam

Printed by:
Ponsen en Looijen - Wageningen