Cold stress and immunity:

Do chickens adapt to cold by trading-off immunity for thermoregulation?

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Cold stress and immunity:

Do chickens adapt to cold by trading-off immunity for thermoregulation?

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Abstract

Future animal husbandry aims at enhanced animal welfare, with minimal use of preventive medical treatments. These husbandry conditions will resemble more natural or ecological conditions. Under such farming systems, animals will experience various kinds of stressors such as environmental (e.g. cold, heat, wind), and social stressors (e.g. pecking in chicken, competition for food). In Western Europe, environmental temperature can drop significantly below the optimal temperature needed for poultry farming during winter season. Apart from cold stress, competition for food could pose nutritional stress in future husbandry practices. Therefore, cold and nutritional stressors can pose a significant threat for poultry farming, as stressors are believed to affect health and welfare of animals. However, effects of cold and nutritional stress on health of poultry are not clearly understood. It has also been proposed that "artificial selection for a trait (e.g. growth, egg production) may program an individual to allocate a large portion of its resources to a demand, leaving it lacking the ability to respond to other demands". Therefore, the focus of this thesis was a) to understand the effects of cold and nutritional stressors on health status (immunocompetence) of two lines of chicken which have selectively been bred for high and low health status (antibody responses). b) to understand the effects of artificially selection on adaptive capacity of chickens to stressful conditions. Important findings of the present thesis are 1. both cold and nutritional stressors did not affect specific antibody responses. 2. both cold and moderate nutritional stressors have positive effect on innate immune component (e.g. phagocytic activity, natural antibody levels), both at cellular and gene levels. 3. cold stress suppresses plasma corticosterone levels in a dose dependent manner, whereas severe nutritional stress enhances plasma corticosterone levels. 4. inverse relation was found between cell mediate immune competence and plasma corticosterone levels. 5. genetic selection for a trait (e.g. selection for either high or low antibody levels) did not affect the immunological adaptive capacity of chickens to both cold and nutritional stressors. It was concluded that cold and nutritional stressors may not pose significant threat for the health of chickens in future farming conditions.

Keywords: Chicken, Cold stress, Feed restriction, Genetic selection, Immune response,

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To my parents and grand parents

Contents

Chapter 1

General Introduction

1.1 Background and relevance of the current thesis to the practical poultry farming

Since the domestication of food animals, animal scientists have concentrated on developing and adapting farming conditions and systems to meet the physiological needs of the food animals. Primarily, this was done to maximize production (egg / meat) and financial profits of farming. The present farming systems which have been developed and standardized for poultry (e.g. battery cages for laying hens) may not have considered the welfare of farm animals. Therefore, future animal husbandry, including poultry aims at enhanced animal welfare, with minimal use of preventive medical treatments [1]. These husbandry conditions may resemble more natural or ecological conditions of the animal. Under such farming systems, animals may have to face various kinds of environmental stressors (e.g. cold, heat, wind) and social stressors (e.g. peck behavior in poultry, competition for food). Apart from facing stressors, animals may have to deal with a higher infection pressure. Higher incidences of bacterial and parasitic infections reported in animals housed outdoors suggest a higher infection pressure on the animals in outdoor farming systems [2]. Furthermore, the ban on usage of in-feed antibiotics in animal husbandry further complicates the situation [3]. In addition, one can not easily ignore the fear that the present breeds of chickens which have undergone intensive selection for higher production may have lost their ability to cope with stress. Therefore, there is a need to understand how environmental stressors affect health status of chickens, and how different breeds of chickens cope with stress.

In general, both internal (e.g. genetic background) as well as external factors (e.g. environmental factors such as temperature, day length, and pathogen) affect the immune system. Most of the studies reported so far have studied the effects of each factor on the immune system separately, whereas few studies exist that include the complex interaction (genotype x environment) effects of both internal and external factors [4-6].

1.2 Cold as a model environmental stress

Among all the environmental stressors, CS induces physiological responses which are of high priority and energy demanding in homeotherms [7]. Furthermore, in Western Europe, environmental temperatures during the winter season can drop well below optimal temperatures required for poultry farming. Therefore, among all physical environmental stressors, CS could significantly affect the health and welfare of poultry in future husbandry

conditions. Even though it is generally accepted that environmental stressors influence the severity of infectious diseases in birds and mammals, their effects on specific immunological functions are relatively unknown [6]. Hypothetical mechanisms through which CS modulates immune responses are depicted in Fig 1. Cold stress can modulate immune responses via two potential pathways 1.Bioenergetic pathway. 2.Endocrine pathway. However, one should keep in mind that these two pathways may not be completely independent from each other.

1. Bioenergetic pathway, if energy costs of mounting immune responses are high as proposed by Demas et al. [8], and both thermoregulation and the immune system are deriving internal energy from the same source, then the increased energy requirement for thermoregulation can limit the internal energy available for the immune system. Thus, resulting limitations in the internal energy for the immune system can detriment the capacity of an individual to mount

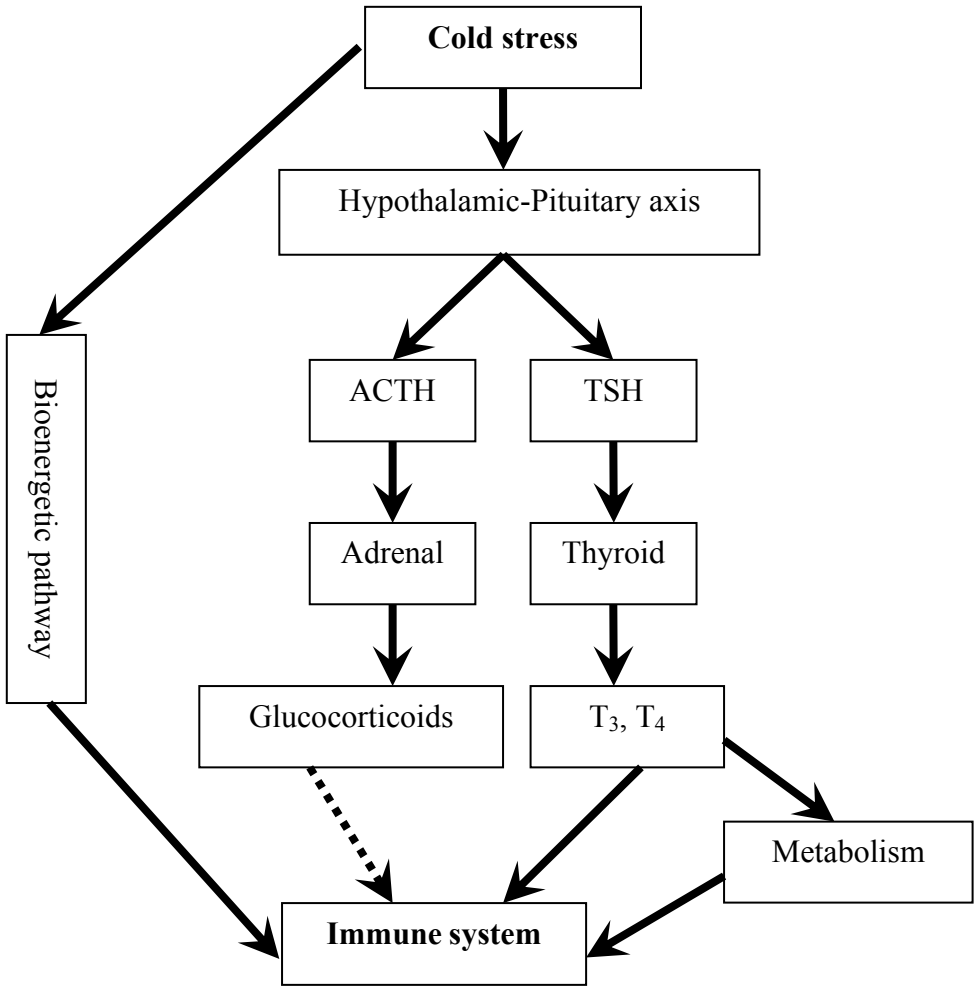

Figure 1. Hypothetical mechanism through which cold stress affects immune system

a proper immune response. 2. Endocrine pathway, cold stress may alter the function of hypothalamic-pituitary axis resulting in changes in the systemic levels of adrenal (e.g. corticosterone) and thyroid hormones levels [e.g. thyroxine (T_4) and tri-iodo-thyronine (T_3)]. As these hormones have immunomodulating effects [9], changes in their levels may affect the immune system either directly or indirectly.

In both chickens and other birds, reported effects of CS on both humoral and cellular immune responses are not consistent. CS enhanced antibody production in broiler chickens [10]. Hester et al. [11] however, reported decreased antibody responses of single-caged hens, but not in hens housed in colony cages after exposure to CS. Regnier et al. [12] found little effects of acute CS on antibody titers to sheep red blood cells in both broiler and layer chickens. Dabbert et al. [13] found no evidence for suppressed humoral immunocompetence following experimentally induced CS in the Northern bobwhite. Whereas, cold exposure had a negative impact on humoral immunocompetence in blue tits [14] and in layer chickens [15]. Cellular immunity was depressed in cockerels exposed to cold [6], whereas an enhancing effect of CS on cellular immunity has been reported in growing layer chicks [1]. Without much experimental evidence, these disagreements between the studies have been attributed to variation in 1. duration of cold exposure, 2. relative time point of measurement of immune parameter to cold exposure, 3. component of immune system measured and 4. genetic background of experimental animals used. Therefore, one of the main aims of the present thesis is to unravel the effects of above mentioned variations.

1.3 Cold stress model as a tool to study adaptive capacity of birds

Adaptive capacity of animals refers to the ability to adjust to environmental variability, extremes, and cope with the consequences of change [16]. The mechanisms underlying adaptation to a stress are largely unknown. The “resource allocation theory” [17] states that various life-history traits such as the immune system, reproductive system, growth, and thermoregulation compete with each other for limited internal resources (e.g. energy, protein). Furthermore, it states that “if the internal resources are limited and if there is an increase in energy requirement for one life-history trait, an individual adapts to such conditions by reallocating internal resources to that life-history trait by compromising the other to maximize fitness”. This suggests that adaptation to stress depends largely on the ability of an animal to

reallocate resources depending on the needs. Therefore, in the present thesis CS has been used to study the adaptive capacity of chickens. It remains to be established whether chickens adapt to CS by increasing energy allocation for thermoregulation by compromising other life-history traits.

1.4 Cold stress model as a tool to study reallocation of resources and trade-offs in evolutionary immunology

In evolutionary and ecological immunology, there has been a growing interest in links between immunocompetence and life-history components such as reproductive effort [18-21], or sexual ornamentation [22, 23]. It is usually assumed that energetically costly immune responses are suppressed during stressful activities such as parental care, and that the released resources from this immunosuppression are then adaptively reallocated to cover other costly activities [20, 24]. So far several researchers have implicitly assumed this when interpreting observed cases of lower immunocompetence measured which correlated with higher reproductive effort or bigger sexual ornaments [19, 22-24]. However, there are not many studies which have experimentally addressed the question about the existence of a resource based trade-off between immunocompetence and other life traits such as thermoregulation and growth and even within the components of the immune system [14]. Furthermore, most ecologists have assessed only one component of the immune system as a measure of overall immunocompetence, assuming that there is a correlated response in the other components of the immune system not measured. As enhancement of one component of the immune system suppress the other [25], suppression of one immune component does not necessarily mean suppression of overall immunocompetence. Therefore, it is possible that the suppressed immune component measured in the above mentioned studies correlating with higher reproductive effort or bigger sexual ornaments may not be due to resource based trade-off.

In homeotherms, thermoregulation can constitute an important energy-demanding constraint to immune function [7]. Therefore, the CS model has been used in the present thesis to investigate immune defence trade-offs with thermoregulation and growth. The energetic trade-offs between vital processes can only be measured after elevating energy requirements for different vital processes under the conditions that exhaust the ability to increase energy acquisition. Otherwise, the extra costs evoked may be fuelled by increased

rates of energy acquisition through feed intake rather than its reallocation from other essential components of energy budget [26]. For this reason, the energy expenditure of growing birds from two selected lines were increased by cold exposure (energy for thermoregulation), and immunization (energy for immune response) while under restricted feeding conditions. This created a unique situation demanding a trade-off between thermoregulation, genetically driven magnitude of immune response and growth. Furthermore, different components of the immune system were monitored to test the hypothesis that "there is also a trade-off between investments in different immune system components".

1.5 Feed restriction as an environmental stress

In the studies described in the present thesis, birds were moderately feed restricted to limit their ability to take up excess feed upon exposure to CS. This helped us to overcome the confounding effect of differences in feeding levels between treatment and control groups with the presence or absence of treatment. On the other hand moderate feed restriction (FR) itself could have act as an additional stress in those studies. Therefore, it was necessary to understand the effect of moderate FR on immune system of chickens. Furthermore, FR has been routinely adapted in the poultry industry for various purposes such as to induce molt [27], to enhance heat stress tolerance [28], and to reduce health and reproduction problems associated with overweight [29]. Nutritional status of an animal has been reported to affect the immune status of an animal [30, 31]. Therefore, many investigators have examined the effect of FR on various parts of the immune system, such as hematological values [32, 33], macrophage function [34, 35], antibody responses [30, 36-39], cell-mediated immunity [30, 36, 39-41] and corticosterone levels [32, 42-44]. It is reported that the expression of physiological functions such as immunocompetence and growth are affected by complex interaction of genetic background, nutrition and environment [38]. However, there have been no reports on the effects of FR on the immunocompetence of layer chickens from different genetic backgrounds. Therefore, it remains to be established whether FR differentially affects immunocompetence in layer chickens with different genetic backgrounds.

1.6 Genetic selection and physiological adaptive capacity

Genetic improvement through artificial selection programs has largely been used as one of the tools to enhance production [45] and to a minor extent to enhance disease resistance in animal production [46]. In poultry, a popular approach has been to divergently select for antibody production against different antigens to enhance disease resistance [46]. Genetic selection for specific antibody responses has been reported to affect other immune responses such as cell mediated immune response [47-49] and natural antibody levels [50]. However, there is not much information available on the effect of genetic selection for antibody responses on physiological adaptive capacity in chickens. It has been proposed that "artificial selection for a trait (e.g. growth, egg production) may program an individual to allocate a large portion of its resources to a demand, leaving it lacking the ability to respond to other demands" [45, 51]. Whether similar is true for chickens which are programmed to have high or low immunocompetence remains to be established. In the present thesis, the above proposal was tested by using chicken lines which are genetically selected either for high or low antibody responses during 21-22 generations. Details regarding the selection process and immunological methods are described in chapter 2. If the above proposal is true then, 1. cold stress should not affect specific antibody responses in either line as they are genetically programmed to mount either high or low specific antibody responses. 2. If mounting specific antibody responses is energy demanding then, birds selected for high antibody responses should have a poor cold stress tolerance when compared with the birds selected for low antibody responses as they are genetically programmed to invest more resources for production of antibodies.

1.7 Aim, scientific importance, hypotheses addressed and outline of the thesis

The main aim of this thesis was to understand the immunological adaptive capacity of chicken lines from different genetic backgrounds to stress. Chicken is one of the most widely introduced vertebrate species on the earth. The nature of distribution of chickens in various climatic conditions, short generation time and availability of vast information on biology of chickens makes it a wonderful experimental animal to understand stress physiology of vertebrates. The knowledge obtained from this thesis should also be helpful to identify strains of chicken most suitable for specific husbandry conditions.

The main aim was achieved by addressing the following hypotheses,

1. "Effects of stress on immunity depend on type of stress, duration of exposure to stress, relative time point of measurement of immunity to the termination of exposure, the immune component measured and the genetic background of the animal".
2. "Genetic selection for a trait (e.g. antibody responses) may program an individual to allocate a large portion of its resources to that demand, leaving it lacking the ability to respond to other demands".
3. "Birds adapt to cold stress by suppressing energy spent for growth and immunocompetence and reallocating the released energy for increased requirement for thermoregulation".
4. "Trade-offs exist between growth, immunocompetence and thermoregulation".

In this thesis, major attention was focused on addressing the first two hypotheses. The data obtained from the experiments carried out to address the first two hypotheses could also be used to address hypotheses 3 and 4. The first hypothesis was addressed in four stages, Stage 1, chicken lines which were selected for mounting either high or low specific antibody responses were subjected to either CS or FR and their effects on immunity were evaluated (chapters 2, 4, 5 & 6). Stage 2, birds from selection lines were subjected to different durations of CS and FR and their ability to mount both innate and adaptive responses was evaluated and described in chapters 2, 4, 5 & 6. Stage 3, birds from selection lines were subjected to CS and were immunized at different time points relative to the termination of CS to measure the effect of CS on relative time point of measurement of immune parameter (chapter 3). Stage 4, use of chicken lines originating from different genetic background and various immune components monitored in all the studies described in chapters 2, 4, 5 & 6, helped us to address the last part of this hypothesis.

In addition to addressing the first hypothesis, to find out the potential impact of CS and FR on the immune system via alteration of neuroendocrine function, plasma adrenal and thyroid hormone levels were quantified and their correlations were tested with the immune parameters and described in chapters 3 and 5.

The second hypothesis was addressed by subjecting chicken lines, which have been divergently selected for antibody responses to CS and described in chapter 6. Artificial selection of these lines for either high or low specific antibody responses has programmed

these animals to divert either more or less energetic resources for production of antibody responses. If the proposed hypothesis is true, then the chickens selected for high antibody responses should divert less energetic resources for thermoregulation and growth.

The third and the fourth hypotheses were tested by simultaneously increasing the energy requirement for thermoregulation and immune responses and measuring the correlated changes in immunocompetence, growth, and body temperature (chapter 6). Apart from addressing those hypotheses, the effects of CS at the gene level was unraveled by quantifying *in vivo* mRNA expression for pro-inflammatory, Th-1 and Th-2 cytokine genes in peripheral blood lymphocytes of chicken lines subjected to CS and is described in chapter 7.

In chapter 8, important findings in this thesis are summarized and discussed.

Chapter 2

Effect of cold stress on immune responses and body weight of chicken lines divergently selected for antibody responses to sheep red blood cells

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2.1 Abstract

Effects of cold stress on the immune system of chicken lines divergently selected for high (H line) and low (L line) antibody responses to sheep red blood cells (SRBC) next to a random bred control (C) line were studied. Twenty-three-days old chicks of the three lines were feed-restricted at 80% *ad libitum*, and subjected to cold stress (CS) at 10^{o}C continuously during 7, 5, 3, 1, or 0 d prior to immunization with keyhole limpet hemocyanin (KLH). We determined specific and natural antibody levels, as a measure of humoral immunity, *in vitro* lymphocyte proliferation as a measure of cell mediated immunity (CMI), reactive oxygen intermediates (ROI) production as a measure of phagocytosis, and body weight gain as a measure of a production trait.

In general, significantly higher antibody responses to all antigens were found in the H line as compared to the other two lines. Antibody responses to KLH were not significantly affected by CS, but an acute transient increase in antibody titers to OVA was found only in H line subjected to 1 d CS. On the other hand, an acute significant increase in antibody titers to LPS was found in C and L line birds subjected to 1 d CS but not in H line. CS enhanced the ROI production. In addition, 7 CS significantly enhanced cellular immune competence measured in vitro, but no significant line effects were found. Body weight gain was negatively affected by CS, especially when CS stress was applied for longer periods.

We conclude that birds respond immediately to cold stress with enhanced innate (phagocyte and NAb) immunity to CS irrespective of their genetic background. When cold stress is prolonged, the cellular adaptive immune response is affected also.

2.2. Introduction

Future poultry husbandry aims at enhanced animal welfare, with minimal use of preventive medical treatments. These husbandry conditions will resemble more natural or ecological conditions. Under such farming systems, animals will undergo various physical and climatic stresses (cold, heat, wind, etc), infectious diseases and social stress. So the animals under such future conditions must be able to cope with, or adapt to the dynamic environments than nowadays, preferably without an increase in production costs and risk of diseases. Because environmental stressors can alter the susceptibility of the animal to infective agents, it is important to learn how stressors affect the immune system of food animals, the adaptive capacity of the animal to respond, and the time it takes for the animal to return to homeostasis. With respect to chickens, results of studies on the effect of cold stress on antibody mediated and cell mediated immune (CMI) responses are not consistent. Cold stress of 7^{o}C enhanced antibody production[10]. However, Hester et al.[11] reported that exposure to 0^{o}C decreased antibody responses of single-caged hens, but not in hens in colony cages. Regnier et al.[12] found little effect of acute cold stress on antibody titers to SRBC. Dabbert et al.[13] found no evidence for suppressed humoral immunocompetence following experimentally induced cold stress in the Northern Bobwhite, *Colinus virginianus*. Whereas, Svensson et al. [14] reported that stress in the form of cold exposure has a negative impact on humoral immunocompetence in Blue tits. CMI was depressed in chickens exposed to cold [6, 52]. Recently, we found enhanced CMI in birds exposed to low temperatures [1]. It is reasonable to suggest that type, and duration of the cold stress treatments, as well as the genetic make up of the birds underlie these divergent results. So far no studies have been reported on the effect of duration of cold stress on both innate and adaptive components of the immune system of genetically selected chicken lines, and their adaptive capacity to cold stress treatments.

In the present study, two lines divergently selected for high (H line) or low (L line) antibody responses to SRBC, next to a non selected chicken line originating from the same parental stock were used. These lines differ with respect to 1) specific humoral immune responses: the H line showing higher antibody titers to various antigens [47, 53], 2) cell-mediated immune responses, the L line showing higher CMI in vitro [47, 48, 54], 3) innate humoral immune responses, the H line showing higher NAb levels [50], and 4) resistance to

infectious diseases [49]. In addition, differences with respect to body weight gain were found, the L line being heavier and growing faster [53, 55].

In the current study, birds of all three lines were restricted-fed at 80% *ad libitum* (160% $ME_{M,}$) with a commercial diet and were subjected to cold stress (10.4±0.5^{o}C) continuously during 7, 5, 3 and 1 days or no stress prior to immunization with KLH. The objectives of this study were fourfold. First, the effect of different durations of cold stress on immunity was studied. Second whether the three lines of chickens, that usually respond differently when immunized under normal conditions, are affected differently when subjected to different durations of cold stress was studied. Third, whether there is a time point during the cold stress that birds have adapted to, or have recovered from the cold stress was studied. Fourth, since immune responses are costly [56], whether the simultaneous stress of restricted feeding and cold stress affects body weight gain and the allocation of resources between growth and immune response was studied.

We measured specific and natural antibodies as a measure of specific and innate humoral immunity, respectively, in three chicken lines subjected to or not subjected to various durations of cold stress prior to immunization. In addition to antibodies, we also measured in vitro lymphocyte proliferation as a measure of cell mediated immunity (CMI), reactive oxygen intermediates (RIO) production as a measure of phagocytosis, and body weight gain as a measure of a production trait.

2.3 Materials and methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Wageningen University.

Chickens

One hundred and eighty-23-d old growing ISA Brown (Warren) medium heavy layer hens from three lines were used in this study. The first two lines were divergently selected for 21 generations for high (H line) or low (L line) primary antibody responses at d 5 after intramuscular (i.m.) immunization with SRBC at 35 d of age. The third line was a random bred control (C) line originating from the same parental line [57]. The C line resembles the genetic pool of the original parental stock of layers [49]

From the day of hatch, until 20 d of age, and subsequently from +15 d after immunization with KLH[1] (40 d of age), chicks were fed ad libitum with a commercial diet (200g kg-1 crude protein, 2600 kcal kg-1 metabolizable energy). Birds were kept according to routine procedures for layer hens in brooder cages.

During the experimental period until the first 15 d after immunization with KLH, birds were restricted fed once a day with the same commercial diet at 80% *ad libitum* (160% of $ME_{M,}$ 120 kcal·$kg^{-.75}$·d^{-1}) [55] for these selection lines. All birds had free access to water throughout the experiment. The light regime was 14 hours light (04.00 till 18.00):10 hours dark. A similar light schedule was maintained when birds were subjected to cold stress. The birds were vaccinated for Marek's disease, and infectious bronchitis at hatch and infectious bursal disease on d 15 of age.

Experimental design

At 23 d of age (experimental day -9), five groups of 12 hens of each (H, C and L) line were randomly assigned to one of five treatment groups, that received a different duration of cold stress. Initially all groups were housed in one climate respiration chamber [58] and maintained at 24.4±0.4°C with relative humidity (RH) of 70.0 ± 0.4% (control temperature) until the cold stress episode. The five treatment groups were subjected to 7 (7 CS), 5 (5 CS), 3 (3 CS), and 1 (1 CS) days of cold stress by transferring the birds to another respiration chamber which was maintained at 10.4 ± 0.5°C (RH of 76.1 ± 1.1%), or were not subjected to cold stress (0 CS). The control group (0 CS) was transported out and back to the same chamber. Immediately after the cold stress episode, all birds were moved back to brooder cages and kept at 22.5 ± 2.2°C (RH of 70 ± 0.2%) until the last experimental day.

At day 0, i.e. 33 d of age, all birds were injected subcutaneously with 1 mg keyhole limpet hemocyanin (KLH, Cal Biochem – Novabiochem Co., San Diego, CA) in 1 ml phosphate buffered saline (PBS, pH 7.2) per bird (Fig. 1). Blood samples were collected from all individual birds at experimental d 0, +1, +8, +11 and +32 after immunization. Specific antibody titers to KLH were determined in plasma obtained at experimental d +1, +8, +11 and +32 after immunization. Blood samples obtained at d +11 were also used to measure phagocytosis. In blood samples collected on d +32, lymphocyte proliferation to both concanavalin A (ConA, Sigma Chemical Co., St. Louis, MO) and KLH were measured. Natural antibody titers to OVA

(Sigma Chemical Co., St. Louis, MO) and LPS (Sigma Chemical Co., St. Louis, MO) were determined in plasma collected on all sampling days. All birds were weighed on experimental d -9, -7, -5, -3, -1, +1, +8, +11 and +32.

Treatment\Days	-9	-7	-5	-3	-1	0	1	8	11	32
7 CS										
5 CS										
3 CS										
1 CS										
0 CS (Control)										
	W	W	W	W	W	B	W+B	W+B	W+B	W+B
						KLH			NBT	LST

Figure 1. Experimental plan: Five groups of 36 hens (12 hens of high, control and low line each) were subjected to cold stress (CS, 10°C) for 7 d (7CS), 5 d (5CS), 3 d (3CS), 1 d (1CS) or 0 d (0CS) before immunization with keyhole limpet haemocyanin (KLH) (1mg/bird) at 33 d of age. All treatment groups were weighed (W) before, during and after CS treatment. Blood samples (B) were collected on d 0, +1, +8, +11 and +32 days. Nitroblue tetrazolium assay (NBT) on day + 11 and lymphocyte stimulation test (LST) on +32 were performed.

Assays

Enzyme-linked immunosorbent assay (ELISA). Antibodies binding to KLH, *Escherichia coli* lipopolysaccharide (*E. coli* LPS), and ovalbumin (OVA) were determined in individual plasma samples obtained from all birds using an indirect two-step ELISA procedure. Plates were coated with either 1 μg/ml KLH, or 4 μg/ml OVA, or 10 μg/ml LPS and after subsequent washing, plates were incubated with serial one-step (KLH and OVA) dilution with an increment of two times or serial two-step (LPS) dilution with an increment of four times of plasma. Binding of antibodies to the antigens was detected using 1:20,000 diluted rabbit anti-chicken IgG_{H+L} labeled with peroxidase (RACh/IgG_{H+L}/PO, Nordic, Tilburg, The Netherlands). After washing, tetramethylbenzidine and 0.05% H_2O_2 were added, and incubated for 10 min at room temperature. The reaction was stopped by adding 2.5 N H_2SO_4. Extinctions were measured with a Multiskan (Labsystems, Helsinki, Finland) at a wavelength of 450nm. The titers were expressed as the 2log values of the highest dilution giving a positive reaction.

Lymphocyte Stimulation Test. An in vitro lymphocyte stimulation test (LST) was performed to determine effects of duration of cold stress on in vitro mitogen, and KLH specific T-cell proliferation. Aliquots of 200 μl whole blood were diluted 1:30 in RPMI tissue

culture medium and cultured during 72 hours at 41°C and 5% CO_2 in a humidified atmosphere. Medium was supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin, in 96-well flat bottom plates, and either 10 μg/ml Con A or 20 μg/ml KLH. The last 12 hours before harvesting, cultures set up in triplicates were pulsed with 0.5 μCi methyl-3H-thymidine (ICNBiomedicals Inc. Aurora, OH). 3H-thymidine uptake by cultures was determined with a Beckman beta-scintillation counter.

Results were expressed as mean counts per minute (CPM) in mitogen or antigen stimulated cultures minus counts per minute in unstimulated cultures (ΔCPM) and as mean stimulation indices (SI). The SI were calculated as SI = counts per minute (CPM) in mitogen or antigen stimulated cultures / CPM in unstimulated cultures.

Reactive oxygen intermediates production assay. The 'Nitroblue Tetrazolium Assay' (NBT) was used to measure reactive oxygen intermediates production as described by Kreukniet et al. [54] with slight modifications. One hundred microliters of 1:60 diluted heparinized blood and 50 μl of 2 mg/mL NBT (Sigma Chemical Co., St. Louis, MO) in PBS were added to 6 wells of 96-well flat-bottom microtiter plates. To the first 3 out of 6 wells with diluted blood and NBT, 10 μl of 1mg/mL of phagocyte stimulator, Zymosan A (prepared from *Saccharomyces cerevisiae,* Sigma Chemical Co., St. Louis, MO) in RPMI was added. To the other 3 wells, 10 μl of RPMI was added. Six additional wells were used as controls, 3 wells out of 6 containing 100 μl of RPMI, 50 μl of NBT in PBS, and 10 μl of Zymosan A was added, and to the other 3 wells, 100 μl of diluted blood, 50 μl RPMI and 10 μl Zymosan A was added. After 1 h of incubation at 41°C, the reaction was stopped with 100 μl 1N HCL. Then each plate was centrifuged and washed two times with PBS (10 min, 850 x g). Finally, 150 μl dimethylsulfoxide (DMSO, Merck, Stutgart, Germany), were added, directly followed by 10 μl 1N KOH. Addition of DMSO caused a color change to the blue spectrum, which was measured with a Multiskan at 690 nm. Mean absorbance of the non-zymosan stimulated samples per bird was subtracted from the absorbance of the stimulated samples, as an indication of the reactive oxygen intermediates production during antigen digestion.

Statistical analysis

Differences in titers of plasma antibodies binding KLH, OVA, and LPS were analyzed by a three-way ANOVA for the effect of treatment, line, time, and their interactions using the repeated measurement procedure using a 'bird nested within treatment and line' option. A two-way ANOVA was performed to determine differences between treatments and lines and their interaction with respect to lymphocyte proliferation to ConA and KLH, superoxide production, and body weight gain (difference between individual body weights on experimental d +1 and d -9). All analyses were according to SAS [59] procedures. Mean differences between treatments and lines were tested with Bonferroni's test.

2.4 Results

Specific antibody responses to KLH

Kinetics of the specific antibody response to KLH in chicks of all three lines subjected to five different time lapses of cold stress are shown in Fig. 2. Highest titers were found at day +8 after immunization in all three lines in most treatment groups. Antibody responses to KLH were affected by an interaction between duration of cold stress, line and time (Table 1). Thus with respect to antibody titers to KLH, the lines responded differently to the duration of cold stress in time. There was no line by treatment interaction or main treatment effect.

Evaluation of the separate lines showed that, on d +1, an enhancement of antibodies binding KLH was found in the H line subjected to 1 CS, 3 CS and 7 CS as compared to the H line birds not subjected to CS. But on d +8 and +11 antibody titers to KLH in CS birds were, though numerically lower than that of the 0 CS group, not significantly different. At +11 d, H line subjected to 3 CS exhibited lower titers to KLH (Fig. 2A) as compared to the 0 CS group of the same line. The C line birds when subjected to CS showed enhanced antibody titers to KLH levels at all time points as compared to the 0 CS birds of the same line, but no CS effects on these time points were found. The response of C line birds subjected to 7 CS to KLH was almost identical to that of non-stressed birds (Fig. 2B). When subjected to cold stress, L line birds showed lower antibody response to KLH when compared with the non stressed L line birds. However, there were no treatment effects within the L line. Titers of the L line birds subjected to 7 CS remained lower until d +32 as compared to the L line birds not subjected CS (Fig. 2C).

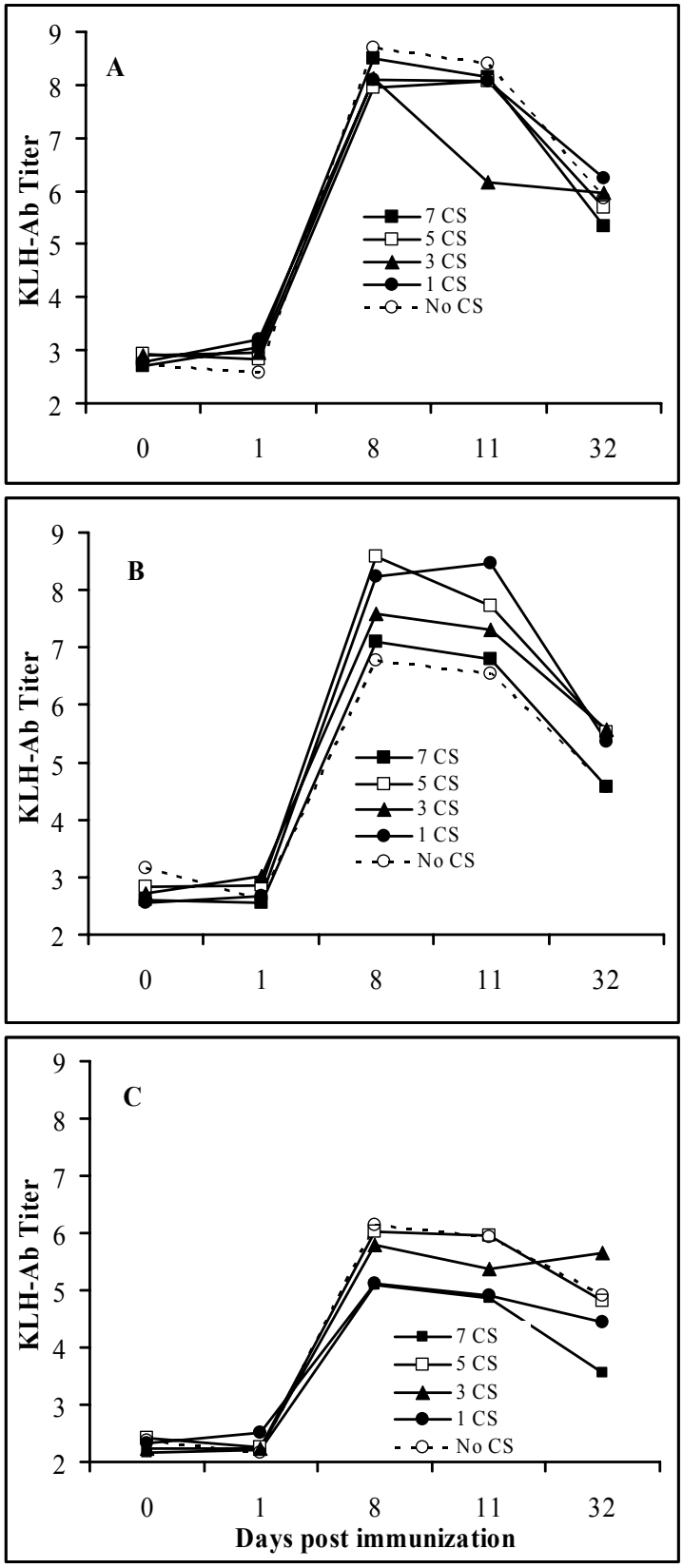

Figure 2. Antibody titers to KLH of high (A), control (B) and low line (C) birds subjected to cold stress (10° C) for 7 d (■), 5 d (□), 3 d (▲), 1 d (●) and no cold stress (○) before immunization with KLH at 33 d of age.

Table 1. Average total serum antibody titers[1,2] from high (H), control (C) and low (L) line hens[3] subjected to one of the five durations of cold stress treatment before immunization with KLH during the complete experimental period

Line	Treatment[4]	Antigen		
		KLH	OVA	LPS
H	7 CS	5.55[2]	2.66[a]	2.91
	5 CS	5.50	2.58[a]	3.30
	3 CS	5.22	2.79[a,b]	3.36
	1 CS	5.68	3.20[b]	3.53
	0 CS	5.65	2.63[a]	3.32
C	7 CS	4.72	2.56	3.56[a,b]
	5 CS	5.46	2.24	3.55[a,b]
	3 CS	5.24	2.72	3.83[a,b]
	1 CS	5.46	2.41	3.92[a]
	0 CS	4.74	2.45	3.13[b]
L	7 CS	3.57	1.65[a]	3.18[a,b]
	5 CS	4.69	2.24[b]	3.49[a]
	3 CS	4.25	2.07[a,b]	3.40[a]
	1 CS	3.86	2.06[a,b]	3.71[a]
	0 CS	4.28	1.95[a,b]	2.57[b]
SEM		0.30	0.21	0.27
Main effects				
Treatment		NS	NS	*
Line		***	***	NS
		H>C>L	H>C>L	
Treatment x line		NS	NS	NS
Time		***	***	***
Time x treatment		*	*	***
Time x line		***	*	***
Time x treatment x line		***	***	*

[ab]Means within treatment and line group with no common superscript differ significantly ($P< 0.05$).

[1]Titers are expressed as the 2log values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of twofold diluted standard positive plasma present on every microtiter plate..

[2]Values are Least Square means ± SEM of the complete experimental period.

[3]Tweleve hens per group (treatment, line).

[4]Birds were subjected to cold stress (10^{o} C) for 7 d (7 CS), 5 d (5 CS), 3 d (3 CS), 1 d (1 CS) and 0 d (0 CS, maintained at 24 ^{o}C) before immunization with KLH at 33 d of age

*P <0.05, ***P <0.001.

Natural antibody (Nab) titers to OVA

A gradual increase with age of levels of NAbs binding OVA were found in all three lines (data not shown). There was no effect of duration of CS on OVA-binding antibodies, but an interaction between treatment, line and time was evident (Table 1). All three lines differed significantly (Table 1) from each other in OVA binding antibody titers. The H line had the highest titers to OVA, followed by the C line, and the L line. There was also no interaction between Treatment and Line. Cold stress induced an acute increase in OVA-binding antibody titers in the H line when subjected to 1 CS only as compared to the H line birds not subjected to CS. When prolonged, CS did not affect titers to OVA. In the C and L line birds, no effects of CS on titers to OVA were found at any time point.

Natural antibody titers to LPS

Plasma antibodies binding *E. coli* LPS were determined in the same plasma samples in which KLH-binding antibodies were determined. Low, but gradually increasing levels of NAbs binding LPS with age were found in all three lines (data not shown). A significant effect (Table 1) of duration of CS on the level of antibodies binding LPS was found. Except for 7 CS, all cold stress treatments induced a significant increase in the levels of antibodies binding LPS in the plasma of L line birds as compared to the L line birds subjected to 0 CS. Also in C line birds, numerically (3, 5, 7 CS), and significantly (1 CS) higher levels of antibodies binding LPS were found as compared to the C line birds subjected to 0 CS. In the H line, titers to LPS were not affected by CS (Table 1). As was true for KLH and OVA, titers of antibodies binding LPS were affected by a time by line by treatment interaction.

In Vitro Lymphocyte proliferation

Stimulation indices of in vitro lymphocyte proliferation to ConA was affected by the duration of CS before immunization (Table 2). Seven day CS enhanced in vitro lymphocyte proliferation to ConA as compared to 0 CS. Whereas 5 CS, 3CS and 1 CS also enhanced the in vitro lymphocyte proliferation as compared to the 0 CS, but not significantly. There was no significant line effect or significant interaction between line and treatment. Specific in vitro lymphocyte proliferation to KLH at day +32 after immunization with KLH was not significantly affected either by the duration of CS, the genetic background of the birds, or the

Table 2. Stimulation indices of in vitro lymphocyte proliferation of whole blood from high (H), control (C) and low (L) line hens[1] subjected to one of the five durations of cold stress treatment before immunization with keyhole limpet haemocyanin (KLH)

Line	Treatment[2]	Con A	KLH
H	7 CS	109.2[a3]	2.3
	5 CS	81.0[a,b]	1.4
	3 CS	54.2[b]	1.5
	1 CS	65.9[a,b]	1.5
	0 CS	48.6[b]	1.7
C	7 CS	128.9[a]	1.6
	5 CS	54.5[b,c]	1.4
	3 CS	98.4[a,b]	1.8
	1 CS	73.1[b,d]	1.8
	0 CS	43.4[c,d]	2.3
L	7 CS	85.7	1.6
	5 CS	90.7	2.0
	3 CS	43.8	1.5
	1 CS	80.1	2.0
	0 CS	55.8	1.8
SEM		19.4	0.3
Main effects			
Treatment		*	NS
Line		NS	NS
Treatment x Line		NS	NS

[a-d]Means within treatment and line group with no common superscript differ significantly ($P< 0.05$).
[1]Tweleve hens per group (treatment, line).
[2]Birds were subjected to cold stress (10^{o} C) for 7 d (7 CS), 5 d (5 CS), 3 d (3 CS), 1 d (1 CS) and 0 d (0 CS, maintained at 24 ^{o}C) before immunization with KLH at 33 d of age
[3]Values are Least Square means of stimulation index ± SEM.
*P <0.05.

interaction between line and treatment (Table 2).

Reactive oxygen intermediates production

Blood samples from all birds collected on d +11 after immunization with KLH were analyzed for reactive oxygen intermediates (ROI) production as a measure of phagocytosis. Cold stress significantly enhanced ROI production of all three lines as compared to the non-stressed group (Table 3). There was no significant difference in ROI production between the three lines. However, there was a significant interaction between cold stress treatment and line. In the H line, a significant increase in ROI production was found in birds subjected to 1

CS and 5 CS as compared to the H line birds subjected to 0 CS. Only when subjected to 3 CS, significantly ($P < 0.05$) higher ROI was found in C line birds as compared to C line birds not subjected to CS, whereas longer or shorter duration of CS than 3 CS did not significantly affect ROI production in the C line birds. The L line birds showed enhanced ROI production when subjected to all CS irrespective of the duration as compared to the L line birds subject to 0 CS. The magnitude of enhancement was, however, less in those L line birds subjected to a longer duration of CS (7 CS).

Table 3. Reactive oxygen intermediates (ROI) production[1] during zymosan A stimulation in the whole blood samples sampled on day +11 after immunization with KLH from high (H), control (C) and low (L) line hens[2] subjected to one of the five durations of cold stress treatment before immunization with keyhole limpet haemocyanin (KLH)

Line	Treatment[3]	ROI production[1] (O.D)
H	7 CS	$0.08^{a,b,c}$
	5 CS	0.17^{a}
	3 CS	0.01^{b}
	1 CS	0.15^{a}
	0 CS	0.02^{b}
C	7 CS	0.06^{a}
	5 CS	0.06^{a}
	3 CS	0.41^{b}
	1 CS	0.08^{a}
	0 CS	0.05^{a}
L	7 CS	0.05^{a}
	5 CS	0.23^{b}
	3 CS	0.10^{a}
	1 CS	0.22^{b}
	0 CS	-0.07^{c}
SEM		0.03
Main effects		
Treatment		***
Line		NS
Treatment x Line		***

[a-c]Means within treatment and line group with no common superscript differ significantly ($P< 0.05$).
[1]Values are Least Square means ± SEM of the mean absorbance (O.D) of the non-stimulated samples subtracted from the absorbance (O.D) of the stimulated samples.
[2]Twelve hens per group (treatment, line).
[3]Birds were subjected to cold stress (10^{o} C) for 7 d (7 CS), 5 d (5 CS), 3 d (3 CS), 1 d (1 CS) and 0 d (0 CS, maintained at 24 ^{o}C) before immunization with KLH at 33 d of age
$P <$ ***<0.001.

Table 4. Average BW[1] and BW gain[2] from high (H), control (C) and low (L) line hens[3] subjected to one of the five durations of cold stress treatment before immunization with keyhole limpet haemocyanin (KLH)

Line	Treatment[4]	BW (g)	BWt gain (g)
H	7 CS	276.8	49.4[a]
	5 CS	293.3	64.6[c]
	3 CS	297.7	75.2[b,c]
	1 CS	302.2	89.4[b]
	0 CS	292.9	89.3[b]
C	7 CS	275.7	41.8[a]
	5 CS	291.0	64.1[b]
	3 CS	307.0	70.4[b,c]
	1 CS	309.3	83.8[c,d]
	0 CS	308.6	93.3[d]
L	7 CS	276.4	39.2[a]
	5 CS	286.9	55.4[a,b]
	3 CS	307.1	65.9[b]
	1 CS	310.4	83.3[c]
	0 CS	298.4	100.3[d]
SEM		18.5	5.71
Main effects			
Treatment		NS	***
Line		NS	NS
Treatment x Line		NS	NS
Time		***	-
Time x Treatment		***	-
Time x Line		NS	-
Time x Treatment x Line		NS	-

[a-d]Means within treatment and line group with no common superscript differ significantly ($P< 0.05$).
[1]values are Least Square means ± SEM of the complete experimental period. [2] BW on 34 d of age minus BW on 24 d of age. [3]Tweleve hens per group (treatment, line).[4]Birds were subjected to cold stress (10^{o} C) for 7 d (7 CS), 5 d (5 CS), 3 d (3 CS), 1 d (1 CS) and 0 d (0 CS, maintained at 24 ^{o}C) before immunization with KLH at 33 d of age. *** P <0.001.

Body weight (gain)

Subjecting chicks to cold stress for different periods before immunization resulted in reduced body weight (numerically) and body weight gain ($P< 0.001$, Table 4). Body weight gain of the birds was inversely related with the duration of cold stress. There was no significant line effect or interaction between treatment and line. There was a significant ($P< 0.001$, Table 4) time effect and time by treatment interaction with respect to body weight. There was no significant interaction between time, line and cold stress treatment.

2.5 Discussion

The future of animal husbandry is aimed to optimize production and enhance animal welfare by minimal use of preventive medical treatments and more naturally based husbandry conditions. Under such farming systems, e.g. 'free range systems', animals will face pressure from different pathogens and/or simultaneously undergo various kinds of physical, climatic, and social stresses. A robust animal under such stressful conditions must be able to cope with, or adapt to these challenges without an increase in production costs or risks of diseases. In this respect, we propose to define the robustness as the capacity of an individual to respond properly to a challenging factor under stress condition. In addition, the robust animal should maintain or return to a response equal to that of an individual, which is similarly challenged but not under stress conditions. It is expected that the genetic background of an individual is an important factor for the adaptation response. Variations in strain, type of cold stress, and type of immune parameter measured may underlie the inconsistent results of the effects of cold stress on immune responses of poultry [10, 11].

The present study was conducted to study the effects of divergent selection of chicken lines for an immune parameter (primary antibody responses to SRBC) on the adaptive response to cold stress, and to measure robustness as defined above. We measured innate and specific immunity and body weight gain in young growing layer chicks subjected to various durations of cold stress. In addition, chicks were restricted-fed to force the birds to set priorities between vital processes [56], in this case thermo-regulation, various immune responses, and growth.

We used layer chicken lines divergently selected for 21 generations for high and low antibody responses to SRBC. These lines differ with respect to various immune responses and body weight gain when measured under normal husbandry temperatures.

The first objective of this experiment was to determine effects of duration of cold stress on parameters of specific and innate immunity. In general, the specific antibody response to KLH was hardly affected by the cold stress treatments. In both H and L lines, antibody titers to KLH were lower in the birds that were subjected to CS, whereas in the C line, higher titers were found in cold-stressed birds. These effects were, however, not significant. Similarly, also the specific CMI to KLH was not affected by the cold stress treatments. On the other hand, cold stress affected parameters of innate immunity, e.g. phagocytosis and natural antibody levels, respectively, with both being enhanced, especially after 1CS. A comparison of the results of this study with a previous study [60] in which chickens were kept for a prolonged period under cold environmental ‘ free range’ conditions, indicates that only a short duration of cold stress affected ROI. In other words, a chronic exposure to low temperature may not significantly affect the cellular part of the innate immunity.

Similarly, mitogen responses to Con A, which combines both features of T cell responses and innate (macrophage derived IL-1) immunity, were enhanced in all birds that were cold stressed. Chickens subjected to the cold stress continuously for 7 CS showed significantly ($P < 0.05$) higher nonspecific cellular immunity to ConA response when compared with the control group (Table 2). This is in agreement with a previous [60], and also suggests that the innate instead of the specific immune system is more sensitive to cold stress. Opposite to the specific antibody response, the mitogenic response was enhanced the most in the H line, but little in the L line. The enhanced mitogen responses to ConA in vitro was measured at day 32 post cold stress treatment. It remains to be established in future studies whether this enhancement reflects the stress effect, or is due to an enhanced rebound response to an earlier decreased mitogen responses to ConA during or shortly after cold stress.

Our second purpose was to indicate the involvement of the genetic background of the birds in the response to the cold stress treatments. The current results showed little or no treatment by line interactions, indicating that the three lines responded similarly to the cold stress treatments, with the exception of the phagocytosis. Only in this assay, difference in response of the three lines to the cold stress treatments was found. Together these results indicate that

divergent selection may have resulted in such a fixation of genes that little variation is left to respond differently to various cold stress treatments in the H and L lines. Effects of cold stress were most pronounced in the immune responses to which the lines had not been selected for: specific antibodies in the L line, CMI in the H line, whereas most variation was still found in the C line. The latter suggests that the lack of difference in responses to cold stress were not due to lack of variation within the original founder line. However, our results suggest that divergent selection for immune response genes did not affect cold stress responses. Future analyses of gene expression during stress in the current lines therefore probably requires identification of different quantitative trait loci than those that are currently developed for measuring immune responses within these lines.

Our third objective was to find a possible time point that the birds of the three lines have adapted to the cold stress environment, in other words: that the birds respond similar to the non cold stressed birds to an antigenic challenge. As discussed above, specific antibodies to KLH were not significantly affected in the three lines with any cold stress treatment. This indicates that the durations of cold stress treatments to the current lines, at this age, and with the level of feed restriction were not severe enough to prevent the birds from all three lines to cope with antigen specific antibody response compared to the non stressed birds. Determination of NAb was included in the current study because 1) Nabs next to complement, form an important part of humoral innate immunity and the first line of defense, 2) they may determine subsequent specific immunity, and 3) with respect to protein content, they constitute a very large part of the antibody repertoire and antigen-binding globulines [61, 62]. With respect to the innate antibodies, only 1 CS treatments enhanced NAb levels in the C and L lines, but the birds of these lines showed an adaptive response to the longer cold stress treatments. All the cold stress treatments resulted in enhanced mitogenic (CMI) responses, that could still be measured at 32 days after ending the cold stress treatments. With the exception of the L line, the other two lines did not show any adaptive response to the cold stress treatments in terms of mitogen responses to ConA.

Our fourth objective was to force the animals to set priorities between vital processes, e.g. thermo-regulation, specific and innate immune responses and body weight gain. It was expected that birds may adapt to the cold condition with for instance energy reallocation away from growth towards thermo regulation. Body weight gain was considered to be a more

appropriate measure rather than the body weight because treatment groups were not balanced weight wise at the beginning of observation period. In the current experiment we did not find significant line differences in body weight gain as reported earlier when the current lines were fed ad libitum [53, 55]. Restriction in the available resources (feed) in the present experiment may be the possible reason for not finding the line differences in terms of body weight gain. Birds subjected to cold stress for more than 2 days (7, 5 and 3 d), showed significantly lower body weight gain than the control group. This seems logical since birds subjected to cold stress may have to spend more energy on thermo-regulation.

Previously we found little effects of humoral immune responses on energy metabolism in growing chicks, and we speculated that combinations of simultaneous climatic stress and restricted feeding may force birds to choose among vital processes with greatest benefits [63]. Furthermore we speculated that innate and cellular immunity as opposed to humoral immunity might be energy demanding as was also proposed by others [64]. Though we did not measure energy (re)allocation within the birds, the lack of cold stress effects on specific humoral immunity, but the enhancing effects on CMI, and the decreasing effects on body weight gain under feed limited conditions can be interpreted in two ways as follows. First, it suggests that after thermo-regulation, innate and CMI have higher priority than humoral immunity and body weight gain, respectively. However, it cannot be excluded that the specific humoral immune response to KLH in the cold stress birds was maintained at the expense of growth. Thus, studies with different restricted feed regimes should reveal reallocation between immune responses, thermo-regulation and growth.

In conclusion, different duration of cold stress did not affect adaptive humoral immunity. Short duration of cold stress enhanced phagocyte activity, and innate humoral immunity (LPS binding antibodies). Longer duration of cold stress was associated with significantly higher in vitro cellular immunity and lower body weight gain. This suggests that birds respond immediately to cold stress with enhanced innate (cellular and humoral) immunity to the non steady state situation, irrespective of their genetic background. Our data suggest that thermo-regulation has prime priority followed by adaptive cellular immunity, humoral immunity, and growth. Further studies are, however, needed to further clarify adaptation to stressful conditions and priority setting for vital life process under stressful conditions.

Chapter 3

Effect of durations of cold stress on plasma adrenal and thyroid hormone levels and immune responses in chicken lines divergently selected for antibody responses

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3.1 Abstract

There is increasing evidence that stress affects various immune processes. Some of these changes are due to hormonal changes like corticosterone (CORT), triiodothyronine (T_3) and thyroxine (T_4). Effects of stress depend on the nature of specific stress (e.g. thermal extremes, diet, pollutant, etc), and stress-modifiers (e.g. genetic make-up, duration and severity of the stressors, etc). We studied the effects of a specific stress (cold stress) with stress-modifiers (duration of stress, and genotype of the bird) on immune responses and plasma adrenal and thyroid hormone levels in three layer-type chicken lines. Two lines were divergently selected for either high (H line) or low (L line) antibody responses to sheep red blood cells (SRBC), the third line was a random bred control (C) line. Three to four week-old growing chicks of the three lines were feed-restricted at 80% *ad libitum*, and subjected to cold stress (CS) at 10^{o}C continuously during 7, 5, 3, 1, or 0 days prior to immunization with keyhole limpet hemocyanin (KLH). Specific antibody titers to KLH, and *in vitro* lymphocyte proliferation (LP) upon mitogen stimulation were measured. In addition, adrenal and thyroid hormone levels were measured in the plasma samples collected at the end of CS.

No significant effect of duration of CS on specific antibody titers was found in all the three lines. A significant enhancing effect of CS was found on LP. A significant dose dependent suppressive effect of CS was found on plasma CORT levels. One day of CS had a significant enhancing effect on T_3 levels. There was no significant effect of duration of CS on T_4 levels. We conclude that CS does not affect specific antibody responses, but CS may have modulating effect on cellular immunity and plasma CORT levels, however depending on the duration of the stress. The present study suggests an inverse relationship between LP and CORT. In addition, this is the first study that reveals an absence of significant differences in adrenal and thyroid hormone levels in the described selection lines.

3.2 Introduction

There are a variety of potential mechanisms by which stressors can alter immune function. One of these mechanisms includes alterations of the endocrine system that in turn modulate immune function [65, 66]. Various studies reported the activation of the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic-adrenal-medullary axis [67-69] and hypothalamic-pituitary-thyroid axis [70] by stressors resulting in hormonal stress responses.

There are reports about the interactions between the thyroid axis and adrenocortical function [71-73]. Moreover, the modulation of immune function by glucocorticoids [74] and thyroid hormones [75] is well known.

It has been hypothesized that the genotype or phenotype of an animal influences its hormonal reaction to stress stimulations, which in turn alters the animal's behavioral adaptability and well being [76, 77]. Understanding a genetic basis for different individual responses to stress is critical in preventing harmful management practices and enhancing productivity in the poultry industry [78, 79].

The impact of stress on the development of an immune response depends on the nature of specific stress (e.g. thermal extremes, diet, pollutant, etc), and stress-modifiers (e.g. genetic make-up, duration, severity, etc) [80]. However this hypothesis has not been tested experimentally. Therefore, the present study was undertaken to analyze the impact of duration of cold stress (CS) on immune responses and their interrelationships with plasma CORT, T_4 and T_3 levels of genetically selected chicken lines for immune responsiveness. In addition we examined whether these differences reflect genetic variation in the plasma CORT, T_4 and T_3 levels in response to CS.

To achieve these objectives, we studied the effects of a specific stressor (cold stress) with stress-modifiers (duration of stress, and genotype of the bird) on plasma CORT, T_4 and T_3 hormone levels, specific antibody responses and lymphocyte proliferative responses in three layer-type chicken lines. Two lines were divergently selected for either high (H line) or low (L line) antibody responses to sheep red blood cells (SRBC), the third line was a random bred control (C) line. The phenotypic differences of two lines have been reported previously [81].

3.3 Materials and methods

Chickens, Housing and Experimental design

The birds, their housing and treatments used in the present experiment have previously been described [81]. Briefly, one hundred and eighty 23d old ISA Brown (Warren) medium heavy layer hens from three lines were used. The first two lines were divergently selected for 21 generations for high (H line) or low (L line) primary antibody responses at d 5 after intramuscular (i.m.) immunization with SRBC at 35 d of age. The third line was random bred, originating from the same parental line, and served as control (C) line [57]. The C line resembles the genetic pool of the original parental stock of layers [49]. These birds were fed once a day at 80% *ad libitum*. Birds had free access to water throughout the experiment. The light regime was 14 h light (04.00 – 18.00):10 h dark.

At 23 d of age (experimental d -9), five groups of 12 hens of each (H, C and L) line were randomly assigned to one of five treatment groups that received a different duration of CS. Birds were allowed to acclimatize to the climate chambers for two days. The five treatment groups were subjected to 7 (7 CS), 5 (5 CS), 3 (3 CS), and 1 (1 CS) d of CS of 10.4 ± 0.5^{o}C (RH of 76.1 ± 1.1%), or no CS (0 CS). Before and after the CS episode, all birds were kept at 22.5 ± 2.2^{o}C (RH of 70 ± 0.2%) until the last experimental day.

At experimental d 0, i.e. 33 d of age, all birds were injected subcutaneously with 1 mg keyhole limpet hemocyanin (KLH, Cal Biochem, Novabiochem Co., San Diego, CA) in 1 ml phosphate buffered saline (PBS, pH 7.2) per bird. Blood samples collected from all individual birds at experimental d 0 (prior to immunization), +1, +8, +11 and +32 after immunization were used to measure various immune parameters [81]. Blood samples collected on d +1 were also used to measure CORT, T_3 and T_4 levels. The experimental protocol was approved by The Institutional Animal Care and Use Committee of Wageningen University.

Assays

ELISA. Antibodies binding to KLH were determined in individual plasma samples obtained from all birds using an indirect two-step ELISA procedure. Plates were coated with 1 μg/ml KLH, and after subsequent washing incubated with serial two-fold dilutions of plasma. Binding of antibodies to the antigens was detected using 1:20,000 diluted rabbit anti-chicken

IgG_{H+L} labeled with peroxidase (RACh/IgG_{H+L}/PO, Nordic, Tilburg, The Netherlands). After washing, tetramethylbenzidine and 0.05% H_2O_2 were added, and incubated for 10 min at room temperature. The reaction was stopped by adding 2.5 N H_2SO_4. Extinctions were measured with a Multiskan (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. The titers were expressed as the 2log values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of two-fold diluted standard positive plasma present on every micro-titer plate.

Lymphocyte Stimulation Test. An *in vitro* lymphocyte stimulation test (LST) was performed to determine effects of duration of cold stress on *in vitro* mitogen stimulated T-cell proliferation. Aliquots of 200 µl whole blood were diluted 1:30 in RPMI tissue culture medium and cultured during 72 hours at 41°C and 5% CO_2 in a humidified atmosphere. Medium was supplemented with 2mM L-glutamine, 100 µg/ml streptomycin, and 100 IU/ml penicillin, in 96-well flat bottom plates, and 10 µg/ml Concanavalin A (Sigma chemical Co., St. Louis, MO). The last 12 hours before harvesting, cultures set up in triplicates were pulsed with 0.5 µCi methyl-^{3}H-thymidine (ICN Biochemicals, Inc., Aurora, OH). ^{3}H-thymidine uptake by cultures was determined with a Beckman beta-scintillation counter.

Results were expressed as mean counts per minute (CPM) in mitogen or antigen stimulated cultures minus counts per minute in unstimulated cultures (ΔCPM) and as mean stimulation indices (SI). The SI were calculated as SI = counts per minute (CPM) in mitogen or antigen stimulated cultures / CPM in unstimulated cultures.

Plasma hormone determinations. Plasma corticosterone was determined using a radioimmunoassay kit (IDS, Inc., Bolton, UK). Plasma 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4) concentrations were measured by radioimmunoassay as described by [82]. Intra-assay coefficients of variation were 4.5 and 5.4% for T_3 and T_4, respectively. Antisera and T_3 and T_4 standards were purchased from Byk-Belga (Belgium).

Statistical analysis. Differences in titers of plasma antibodies binding KLH were analyzed by a three-way ANOVA for the effect of cold treatment, line, time, and their interactions using the repeated measurement procedure using a 'bird nested within treatment and line' option. A

Table 1. Average total plasma antibody titers[1] binding keyhole limpet haemocyanin (KLH) and stimulation indices of *in vitro* mitogen-stimulated lymphocyte proliferation of whole blood from 3 layer-type chicken lines subjected to 1 of 5 durations of cold stress treatment before immunization with KLH (adopted from chapter 1)

Line[2]	Treatment[3]	Antibody titers	Stimulation index[4]
High (H)	7 CS	5.55	109.2^{a}
	5 CS	5.50	81.0^{ab}
	3 CS	5.22	54.2^{b}
	1 CS	5.68	65.9^{ab}
	0 CS	5.65	48.6^{b}
Control (C)	7 CS	4.72	128.9^{a}
	5 CS	5.46	54.5^{bc}
	3 CS	5.24	98.4^{ab}
	1 CS	5.46	73.1^{bd}
	0 CS	4.74	43.4^{cd}
Low (L)	7 CS	3.57	85.7
	5 CS	4.69	90.7
	3 CS	4.25	43.8
	1 CS	3.86	80.1
	0 CS	4.28	55.8
SEM		0.30	19.4
Main effects			
Treatment (T)		NS	*
			$0 \leq 3 \leq 1 \leq 5 \leq 7$
Line (Li)		***	NS
		H>C>L	
T x L		NS	NS
Time (Ti)		***	-
Ti x T		*	-
Ti x Li		***	-
Ti x T x Li		***	-

[a-c]Means within a parameter and line group with no common superscript differ significantly ($P < 0.05$).
[1]Titers were expressed as the 2log values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of two-fold diluted standard positive plasma present on every microtiter plate.
[2]For line and treatment, n= 12 hens per group.
[3]7CS, 5CS, 3CS, 1 CS = birds subjected to cold for 7, 5, 3, 1 d before immunization, respectively; 0CS = birds were kept in climate chamber maintained at 22.5 ± 2.2°C.
[4]Values are Least Square means of stimulation indices. *P <0.05, *** P <0.001.

two-way ANOVA was performed to determine differences between treatments and lines and their interaction with respect to lymphoproliferation, corticosterone, T_3 and T_4 levels. Simple correlation between different immune parameters, corticosterone, T_3 and T_4 were calculated using Pearson's correlation coefficients. All analyses were according to SAS procedures [59]. Mean differences of treatment and line were tested with Bonferroni's test.

3.4 Results

Specific antibody responses to KLH.

Results of specific antibody responses to KLH have been published before [81]. Briefly, no significant effect of duration of CS on antibody responses to KLH was found (Table 1). There was a significant line effect, the H line had the highest antibody titers followed by the C and the L line.

In Vitro Lymphocyte proliferation.

Results of in vitro lymphocyte proliferation have been published before [81]. Briefly, stimulation indices of in vitro lymphocyte proliferation to ConA were significantly affected by the duration of cold stress before immunization (Table 1). Seven days of CS significantly enhanced in vitro lymphocyte proliferation to ConA as compared to 0 CS. In addition, higher, but not significantly different lymphocyte proliferation was also found in birds subjected to 5 CS, 3 CS and 1 CS.

Adrenal hormone levels.

Cold stress significantly depressed plasma CORT levels, and this depressive effect was more pronounced with increasing duration of exposure to CS (Table 2). All three lines responded similarly to the CS as there was no line effect or line by CS treatment effect (Table 2).

Thyroid hormone levels.

One day of CS prior to immunization resulted in a significant increase in circulating T_3 concentrations in plasma compared to levels of 0 CS group, whereas longer duration of CS did not significantly affect T_3 concentrations. No significant line effect or interaction term

was found (Table 2). T_4 concentrations were not significantly affected by duration of CS, line or interaction between CS treatment and line (Table 2). Effect of genetic selection on adrenal and thyroid hormone levels. Genetic selection for high or low primary antibody response did not significantly affect the plasma CORT, T_3 and T_4 levels in the described selection lines (Table 3).

Table 2. Corticosterone (CORT), 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4) hormone levels[1] in plasma samples from high (H), control (C) and low (L) line hens subjected to 1 of 5 durations of cold stress prior to immunization with keyhole limpet haemocyanin (KLH)

Line[2]	Treatment[3]	CORT	T_3	T_4	T_3 / T_4
H	7 CS	8.16	0.35^{ab}	9.38^{cb}	0.042
	5 CS	16.10	0.23^{a}	22.39^{a}	0.012
	3 CS	15.45	0.96^{ab}	15.02^{ac}	0.080
	1 CS	13.59	1.10^{b}	5.78^{b}	0.190
	0 CS	14.39	0.83^{ab}	8.65^{cb}	0.104
C	7 CS	11.05^{a}	0.25^{a}	14.78	0.027
	5 CS	13.91^{ab}	0.16^{a}	10.25	0.018
	3 CS	10.36^{a}	0.51^{a}	9.86	0.062
	1 CS	15.90^{ab}	2.02^{b}	7.17	0.294
	0 CS	25.84^{b}	0.53^{a}	8.79	0.064
L	7 CS	9.38^{a}	0.17^{a}	8.57	0.020
	5 CS	11.68^{a}	0.65^{ab}	6.96	0.093
	3 CS	20.47^{ab}	0.47^{ab}	6.19	0.073
	1 CS	22.62^{ab}	1.36^{b}	7.77	0.184
	0 CS	32.62^{b}	0.58^{ab}	9.07	0.062
	Pooled SEM	5.55	0.36	3.28	0.041
	Main effects				
	Treatment	*	***	NS	***
		$0 \geq 1 \geq 3 \geq 5 \geq 7$	$1 > 0 \geq 3 \geq 5 \geq 7$		
	Line	NS	NS	NS	NS
	Treatment x Line	NS	NS	NS	NS

[a-c]Means within a parameter and line group with no common superscript differ significantly ($P < 0.05$).
[1]Values are least square means of hormone levels (ng /mL).
[2] For treatment, line, n = 5 hens per group.
[3]7CS, 5CS, 3CS, 1CS = birds subjected to cold for 7, 5, 3, 1 d before immunization, respectively; 0CS = birds were kept in climate chamber maintained at 22.5 ± 2.2°C.
*P < 0.05, ***P < 0.001.

3.5 Discussion

In the present study, the effect of different durations of CS on specific antibody and in vitro lymphocyte proliferative responses and their relationship with plasma CORT, T_4 and T_3 levels in genetically selected chicken lines were studied. There was no significant effect of duration of CS on specific antibody responses to KLH. Phenotypic differences in specific antibody responses in the present lines of chicken were not altered by the duration of CS. On the other hand, the duration of CS significantly enhanced mitogen induced lymphocyte proliferation. This finding is in agreement with our previous finding with the same selection lines, which mounted higher in vitro mitogen responses when kept in the cold under free range conditions [1], and with others [83]. However, our finding contradicts the reported suppressed humoral immunity and lymphocyte proliferation reported in rats subjected to CS [84] and suppressed CMI reported in chickens subjected to CS [6].

Table 3. Corticosterone (CORT), 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4) hormone levels[1] at 23 d of age in plasma samples from high (H), control (C) and low (L) line hens divergently selected for antibody responses

Line[2]	CORT	T_3	T_4
High	37.2	1.2	5.0
C	37.9	1.1	6.0
L	42.7	1.6	6.3
SEM	6.0	0.2	0.6
Line effect	NS	NS	NS

[1]Values are Least Square means of hormone levels (ng / ml).
[2]n = 20 hens per line.

Stress is known to be related to neuro-chemical and hormonal changes including alterations in adrenal and thyroid hormone levels [85, 86]. The interaction between adrenal and thyroid axis with the immune system, on the basis of either existence of adrenal and thyroid receptors on lymphocytes as well as the frequent immune alteration associated with

physiological and pathological fluctuation of adrenal and thyroid hormones, was suggested. Over the past years, the strong evidence of participation of the neuroendocrine system in the modulation of humoral immunity [87, 88] and lymphocyte activity [65, 66, 87, 89] have been accumulated.

Glucocorticoids are considered as being immunosuppressive [90]. Several studies have also revealed immune enhancing [91] and immunomodulating effects of glucocorticoids [92]. Moreover, it was demonstrated that lymphoid cells possess glucocorticoid receptors and therefore are sensitive for glucocorticoid action [93]. In the present study, exposing birds to different duration of CS suppressed the CORT levels in a dose dependent manner. The results of CORT and lymphoproliferation taken together suggest an inverse relation. Considering the results of corticosterone and lymphoproliferation suggests that the intensity of CS used in the present experiment is mild. Siberman et al. [70] reported an early increment of CORT levels that returned to normal values after 3 weeks in mice exposed to a chronic mild stress. Ayensu et al. [94] reported higher CORT levels in rats after 4 weeks of chronic mild stress. However, others have not found elevated levels of this hormone neither in human nor in animals after prolong stress situations [69]. Moreover, it was demonstrated that chronic stress induces a hypersuppressive state for induced CORT secretion in response to acute stress, which is caused by partial habituation, coping and adaptation to the stressors [95].

There is a close interaction between the thyroid axis and the HPA axis. It has been reported that both T_3 and T_4 influence plasma and adrenal corticosterone levels [72], apart from that T_3 and T_4 also have direct immunomodulating activities [75]. Moreover, chronic stress has been generally associated with a suppression of thyroid axis function. During stress, a suppressed secretion of thyroid stimulating hormone (TSH) and decreased conversion of T_4, the relatively inactive to T_3 was described [96]. Siberman et al. [70] described that chronic stress exposition was able to induce a reduction of thyroid hormone levels in mice. Therefore, we determined T_3 and T_4 levels in the same samples in which CORT was determined. Birds exposed to the shortest duration of CS prior to immunization showed significantly enhanced T_3 levels, whereas birds subjected to the longer duration of CS did not affect the T_3 levels. The increased circulating T_3 levels in chickens exposed to mild cold period [97] could be due to an enhanced conversion of T_4 to T_3, a process which is known to be sensitive to ambient temperature [98]. Apart from that the significant increase in plasma T_3 levels after 1 CS

suggests an increase in metabolic heat production. However, prolonged CS results in acclimation and restoration of heat balance as can be inferred based on normalized plasma T_3 levels as well as progressively decreased plasma CORT levels.

In the present study there was no significant effect of CS on T_4 levels. A clear decrease in lymphoid proliferative response to mitogens and a depression of primary humoral immune response in hypothyroid animals were reported [99]. However, the effect of hyperthyroidism provoked by T_3 or T_4 administration on humoral and cellular immunity is still controversial [70]. The lack of correlation between T_3 or T_4 levels and proliferative responses in the present study suggests that the effect of CS on proliferative responses is unrelated to changes in T_3 or T_4 levels.

In addition, we also studied the effect of genetic selection of chicken lines for antibody responses on levels of plasma CORT, T_3 and T_4. This study revealed an absence of significant differences in adrenal and thyroid hormone levels in the described selection lines. Similar results were reported in broilers selected for high or low fat content [100]. However, differences may exist in daily rhythms in concentration of hormones in plasma or even with life stage of the selection lines.

In conclusion CS does not affect specific antibody responses. But CS has modulating effect on cellular immunity and plasma CORT levels, however depending on the duration of the stress. There were no direct correlations between any immune parameters and the adrenal or thyroid hormone levels. However, results of CORT and lymphocyte proliferation taken together suggest an inverse relation. In addition, this is the first study that reveals an absence of significant differences in adrenal and thyroid hormone levels in the described selection lines. However, further studies are needed to confirm the effect of the CS on hormone levels, immune performance and their interactions.

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Chapter 4

Duration of cold stress modulates overall immunity of chicken lines divergently selected for antibody responses

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4.1 Abstract

Effects of different duration of cold stress (CS) and time point of immunization relative to the CS of three chicken lines were studied. First two chicken lines were divergently selected for high (H) and low (L) antibody responses and the third was a random bred control (C) line. In two experiments, 26 d old growing chicks of the 3 lines were feed-restricted at 80% of *ad libitum*, and were subjected to CS of 10^{o}C during 7, 2, or 0 d. Birds were immunized with keyhole limpet hemocyanin (KLH) at -1, +1, +3, +5 or +7 d relative to the end of the CS treatment. Specific antibodies to KLH were determined. In addition, *in vitro* lymphocyte proliferation to concanavalin A (ConA) and KLH as measures of cell mediated immunity (CMI), Zymosan-induced reactive oxygen intermediates production as a measure of phagocytosis, and BW gain as a measure of a production trait were determined.

Significantly higher antibody responses to KLH were found in the H line as compared to the other 2 lines. Specific antibody responses to KLH were not significantly affected by the CS treatments. CS had delayed effect on *in vitro* mitogen responses to ConA. *In vitro* lymphocyte proliferation responses to ConA were higher in the L line birds than in the other 2 lines. In general 7 d of CS significantly enhanced cellular immunity to ConA, whereas 2 d CS treatment had differential effects on lymphocyte proliferation to ConA, depending on the line of bird and time point of immunization. KLH-specific lymphocyte proliferation was enhanced by 2 d of CS at 28 d after immunization. Effects of various CS treatments and the time point of immunization on the production of reactive oxygen intermediates were inconsistent. In addition, BW gain was negatively affected by CS.

We conclude that the innate part of the immune system (phagocytes), responded immediately to CS with an as yet unexplained variability, irrespective of the genetic background. When CS is prolonged, the cellular adaptive immune response and to some extent the specific humoral immune response are also affected. The lack of line-by-treatment interactions suggests that the genetic background was a prominent factor for the magnitude of the specific immune response. Our data confirmed earlier studies that under restricted feeding with simultaneous stress (energy demand for thermoregulation and growth), cellular immunity is more sensitive than humoral immunity. A negative correlation BW gain and cellular immunity suggest a trade-off between these 2 life traits.

4.2 Introduction

The physical environment of an animal exerts considerable effects on the immune status, particularly the overall capacity to mount immune responses, and as a consequence protection of the host to infectious disease [101]. Under future farming systems resembling more natural conditions, animals will face various kinds of simultaneous environmental stressors [1]. Environmental stressors like temperature, light, air quality, infective agents, environmental contaminants, etc. are known to affect immune responses of poultry [101]. In homeotherms, during winter season thermoregulation is probably an important energy demanding process, and therefore may be a constraint to immune function. Hence, it is usually assumed that energy-demanding immune responses are suppressed during the winter season. Resources may then be reallocated to cover other energy demanding life traits like thermoregulation. Likewise, in wild birds, energy demanding processes such as breeding, brood care, migration, or having sexual ornaments are usually accompanied by decreased immune responses and decreased health status, especially during cold seasons (reviewed in [56]). In both chickens and other (wild life) birds, the effect of cold stress (CS) on both humoral and cellular immune responses is not consistent. CS of 7^{o}C enhanced antibody production [10]. Hester et al. [11] reported decreased antibody responses of single-caged hens, but not in hens in colony cages after exposure to 0^{o}C. Regnier et al. [12] found little effect of acute CS on antibody titers to SRBC. Dabbert et al. [13] found no evidence for suppressed humoral immunocompetence following experimentally induced CS in the Northern bobwhite. Whereas, Svensson et al. [14] reported that stress in the form of cold exposure has a negative impact on humoral immunocompetence in Blue tits. Cellular immunity (CMI) was depressed in chickens exposed to cold [6]. We recently reported enhanced CMI in growing layer chicks exposed to low temperatures [1, 81], whereas CS did not affect specific antibody responses in cold stressed chicks [81]. In the later study, we found, however, a differential effect of different duration of CS on the cellular immune system. We predicted that the effect of CS on immune responses may depend on the time point after the stressor at which immune responses are determined, next to the duration of CS [81]. In addition, since it was proposed that trade-offs exist between components of the immune system, and also between the immune system and other life traits [102], it is expected that the genetic make up of the birds, e.g. immune responsiveness, may affect responses to stress conditions as well.

In the present experiments, two lines divergently selected for high (H line) or low (L line) antibody responses to SRBC, next to a non selected chicken line originating from the same parental stock were used. These lines differ with respect to 1) specific humoral immune responses: the H line showing higher antibody titers to various antigens [47, 53], 2) cell-mediated immune responses, the L line showing higher CMI *in vitro* [47, 48, 54], 3) innate humoral immune responses, the H line showing higher NAb levels [103], and 4) resistance to infectious diseases [49]. In addition, differences with respect to body weight gain were found, the L line being heavier and growing faster [53, 55].

Two experiments were conducted to study the effects of duration of CS, time of immunization relative to the end of CS and genetic background of chicken lines on immune responses.

The objectives of the two experiments were threefold. First, the effects of duration of CS on humoral and cellular immune responses of selected chicken lines were studied. Second, we studied whether the time point of immunization relative to the end of CS affected immune responses of these lines. Third, we used chicken lines selected for antibody responses to establish a genetic basis for possible trade-offs between components of the immune system, and between the immune system and body weight gain under simultaneous stress, e.g. energy demand for thermoregulation, immune responses and growth. In addition, selection lines offer the opportunity to define the importance of either genetic background or the environment for gene expression.

4.3 Materials and methods

Chickens, housing and feed

In both experiments 26-d-old growing ISA Warren Brown medium heavy layer hens from three lines were used. Two lines were divergently selected for 21 generations for either high (H line) or low (L line) primary antibody responses at day 5 after intramuscular (i.m.) immunization with SRBC at 35 days of age. The third line was a random bred control originating from the same parental line, and served as control (C) line [57]. The C line resembles the genetic pool of the original parental stock [49].

Birds were fed *ad libitum* from the day of hatch, until 26 d of age (experimental day -9), and subsequently from 50-53 d of age (experimental day +15) with a commercial diet (200 g

kg^{-1} crude protein, 2,600 kcal kg^{-1} ME). Birds were group housed in brooder cages from the day of hatch until the last experimental day.

During the experimental period (experimental day -9, until experimental day +15), birds were fed once a day with the same commercial diet at 80% of *ad libitum* (160% of MEm, i.e.120 kcal·$kg^{-.75}$·d^{-1}, previously established for these selection lines [55]. All birds had free access to water throughout the experiment. The light regime was 14 hours light (04.00 till 18.00):10 hours dark. A similar light schedule was maintained when birds were subjected to CS. The birds were vaccinated for Marek's disease and infectious bronchitis at hatch and infectious bursal disease on day 15 of age. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Wageningen University.

Experimental design

Experiment 1. Two days of CS at various time points prior to immunization. Effects of 2 days of CS at different time points prior to immunization on immune responses of the three lines were studied using a 3 x 4 factorial design of treatments. Factors were line and time points of CS prior to immunization with KLH. At 26 days of age (experimental day -9), groups of 12 hens of each line (H, C and L), were randomly assigned to one of the four treatment groups, that received 2 days stress either at 8, 3 or 1 days prior to immunization or no CS prior to immunization on day 0. (i.e. 35-37 days of age). All birds were injected sc with 1 mg keyhole limpet hemocyanin (KLH, Cal Biochem – Novabiochem Co., San Diego, CA) in 1 ml phosphate buffered saline (PBS, pH 7.2) per bird. Treatment groups were subjected to CS by transferring the birds to one of two climate respiration chambers [58]. Birds were allowed to acclimatize to the climate respiration chamber for a day and then the temperature was lowered to 10.4 ± 0.5^{o}C (RH of 76.1 ± 1.1%) for 2 days (2 CS). The control groups (0 CS) were also transported and kept in the same climate chambers for 2 days, which were used to give CS but the chamber temperature was maintained at 24.0^{o}C. Blood samples were collected from all birds at days 0, +1, +7, +10 and +28 after immunization. Specific antibody titers to KLH were determined in the plasma samples collected at day 0, +1, +7 and +10. Blood samples obtained at day +7 were also used to measure production of reactive oxygen intermediates (ROI) *in vitro*. In blood samples collected on day +28 after immunization, *in vitro* lymphocyte

proliferation to concanavalin A (ConA, Sigma Chemical Co., St. Louis, MO) and KLH were determined. All birds were weighed on experimental days -9 and +2 after immunization.

Experiment 2. Two or 7 days of CS prior to immunization at any of the five different points. Effects of 2 (2 CS) or 7 (7 CS) days of CS on immune responses of the three lines were studied using 3 x 3 x 5 factorial design of treatments. Factors were line, CS duration, and time points of immunization after CS. At 26 days of age (experimental day -9), groups of 10 hens of each line were randomly assigned to one of fifteen treatment groups, that received either 7CS, 2CS or no CS (0CS) and immunized at one of five different time points after the CS treatment. Birds were immunized with 1 mg of KLH in PBS either on day -1, +1, +3, +5, or +7 (i.e. 34 - 42 days of age) after 7CS, 2CS or 0CS. From the day of hatch until the last experimental day all birds were housed in one of the two climate respiration chambers and maintained at 21.8 ± 1.4^{o}C with relative humidity (RH) of 56 ± 14% until the CS episode. Treatment groups were subjected to 7CS, or 2CS by transferring the birds to a similar climate respiration chamber which was maintained at 10.2 ± 0.9^{o}C (RH of 69 ± 3.5%), or were not subjected to CS (0CS). The 0CS group was transported out of the chamber and back to the same chamber to mimic the transportation effect

Blood samples were collected from all individual birds at days 0, +1, +7, +10 and +28 after immunization. Specific antibody titers to KLH were determined in plasma obtained at days 0, +1, +7, and +10 after immunization. Blood samples obtained at day +7 were also used to measure ROI production. In blood samples collected on day +10 after immunization *in vitro* lymphocyte proliferation to concanavalin A (ConA,) was determined. Samples collected on day +28 (day 20-28) after immunization were used to measure *in vitro* lymphocyte proliferation to ConA and KLH. All birds were weighed on experimental days -7, and 0.

Assays

ELISA. Antibodies binding to KLH were determined in individual plasma by indirect two-step ELISA as described before in chapter 2.

In vitro Lymphocyte Proliferation to ConA and KLH. In vitro lymphocyte stimulation tests (LST) of whole blood were performed as described in chapter 2.

Nitroblue Tetrazolium Assay (NBT). The NBT assay was used to determine reactive oxygen intermediates production (ROI) by phagocytes in whole blood as described in chapter 2.

Statistical analysis

Experiment 1. Differences in titers of plasma antibodies binding KLH, and also body weight were analyzed by a three-way ANOVA for the effect of time point of CS prior to immunization, line, time, and their interactions by the repeated measurement procedure using a 'bird nested within treatment and line' option. Nested term was used as an error term to test effects of treatment, line and their interaction, whereas time and its interactions were tested against the residual error. A two-way ANOVA was performed to determine differences between treatments and lines and their interaction with respect to lymphocyte proliferation to ConA and KLH, ROI production, and body weight gain (difference between individual body weights on experimental day +2 and day -9 relative to the end of CS).

Experiment 2. Differences in titers of plasma antibodies binding KLH were analyzed by a four-way ANOVA for the effect of duration of CS, line, time point of immunization relative to the end CS, time, and their interactions by the repeated measurement procedure using a 'bird nested within duration of CS, time point of immunization and line' option. Nested term was used as an error term to test the effects of duration of CS, immunization, line effects and their interactions, whereas time and its interactions with duration of CS, immunization and line were tested against the residual error. A three-way ANOVA was performed to determine differences between duration of CS, time point of immunization and lines and their interaction with respect to lymphocyte proliferation to ConA and KLH and ROI production. A two-way ANOVA was performed to determine differences between duration of CS, line and their interaction at a time point of immunization with respect to body weight gain (difference between individual body weights on experimental day 0 and day -7 relative to the end of CS).

All analyses were according to SAS [59] procedures. Mean differences between duration of CS, time point of immunization and lines were tested with Bonferroni's test.

Table 1. Immune parameters determined in high (H), control (C) and low (L) line hens[1] subjected to cold stress (CS) at 1 of 3 time points prior to immunization with keyhole limpet haemocyanin (KLH) in experiment 1

Line	Treatment[2]	KLH-Ab[3]	ConA[4]	KLH[4]	ROI[5]
H	8 d p.i^{w}	5.46	33.62^{a,c}	2.77^{a}	0.47^{a}
	3 d p.i^{x}	5.83	8.98^{a}	0.98^{b}	0.30^{b}
	1 d p.i^{y}	6.03	71.63^{b}	3.83^{a}	0.25^{b}
	0 CS^{z}	6.08	43.05^{c}	1.07^{b}	0.33^{b}
C	8 d p.i^{w}	4.87^{ac}	82.48^{a}	3.02^{a}	0.40^{a}
	3 d p.i^{x}	5.63^{b}	10.75^{b}	1.06^{b}	0.23^{b}
	1 d p.i^{y}	4.51^{c}	56.36^{a,c}	3.79^{a}	0.45^{a}
	0 CS^{z}	5.77^{b}	32.73^{c}	0.90^{b}	0.52^{a}
L	8 d p.i^{w}	3.76	72.89^{a}	2.83^{a}	0.26^{a}
	3 d p.i^{x}	4.32	29.51^{b}	1.71^{a,b}	0.23^{a}
	1 d p.i^{y}	4.11	50.20^{a,b}	2.09^{a,b}	0.41^{b}
	0 CS^{z}	3.82	49.31^{a,b}	1.10^{b}	0.20^{a}
	SEM	0.25	9.44	0.41	0.05
	Main effects[7]				
	T	*	***	***	***
		x≥z≥y≥w	w≥y≥z≥x	y≥w≥x≥z	w≥y≥z≥x
	L	***	NS	NS	***
		H>C>L			C>H≥L
	T x L	*	**	*	***

[a-c]Means within a parameter and line group with no common superscript differ significantly ($P< 0.05$).
[1]Twelve hens per group (treatment, line).
[2]Birds subjected to CS (10^{o}C) for 2 d at 8 d prior to immunization (8 d p.i.), 3 d (3 d.p.i), or 1 d (1 d.p.i) with KLH versus a group of birds kept at 24 ^{o}C continuously (0 CS).
[3]KLH binding antibody titers. Values are least square means of the complete experimental period Titers were expressed as the 2log values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of two-fold diluted standard positive plasma present on every microtiter plate.
[4]Stimulation indices of *in vitro* lymphocyte proliferation to concanavalin A (ConA) and KLH of whole blood at d + 28 after immunization.
[5]Production of reactive oxygen intermediates (ROI) production during zymosan A stimulation in the whole blood samples sampled on d 8 after immunization with KLH. Values are the least square means of the mean absorbance of the non-stimulated samples subtracted from the absorbance of the stimulated samples.
[6]T = treatment effect, L = line effect.
*P <0.05, ** P <0.01, *** P <0.001.

4.4 Results

Specific antibody responses to KLH

Experiment 1. Subjecting three lines of chickens to CS at 8, 3, or 1 day prior to immunization (d.p.i) did not significantly affect antibody titers to KLH in the H and the L line birds (Table 1). However, CS significantly suppressed antibody titers to KLH in the C line birds, which had been subjected to CS at 8 and 1 d.p.i. All three lines significantly differed from each other. The H line birds had significantly ($P< 0.001$) higher titers of KLH binding antibodies than the C and the L line birds (Line effect).

Experiment 2. Subjecting three lines of chickens to different duration of CS affected specific antibody titers to KLH. Birds subjected to 2CS had highest antibody titers to KLH followed by 0CS and 7CS. A significant line effect was found. The H line birds had highest antibody titers to KLH followed by the C and the L line birds (Table 2). There was a significant effect of time point of immunization relative to the end of CS. The shorter the duration of time birds had after CS, the lower the antibody titers to KLH (Table 2). Antibody responses to KLH were not significantly affected by any interactions among duration of CS, line and time point of immunization (Table 2).

In Vitro Lymphocyte proliferation to ConA

Experiment 1. Lymphocyte proliferation to ConA was affected by a line by treatment interaction (Table 1). There was a significant effect of CS on the SI of *in vitro* lymphocyte proliferation to ConA, but there was no line effect. The H line birds subjected to CS at 1 d.p.i showed significantly enhanced SI when compared with the 0CS group of the same line. On the other hand, the H line birds subjected to CS at 3 d.p.i. showed significantly suppressed SI to ConA when compared with the 0CS (Table 1) group. The C line birds subjected to CS at 3 d.p.i. showed significantly suppressed SI to ConA when compared with the C line birds subjected to 0CS. On the other hand, the C line birds subjected to CS at 8 d.p.i. showed significantly enhanced SI when compared with respective control group. CS at different time points prior to immunization did not significantly affect SI of the L line birds, when compared with the L line birds subjected to 0CS.

Table 2. Average total serum keyhole limpet haemocyanin (KLH)-binding antibody titers[1] from high (H), control (C) and low (L) line hens[2] immunized at 1 of 5 time points after exposure to 2, 7, or 0 d of cold stress (CS) before immunization with KLH during the complete experimental period in experiment 2

		Time point of immunization after CS					
Line	Treatment[3]	-1	+1	+3	+5	+7	
H	7 CS^{x}	6.81[2]	6.80	7.38	7.35	7.16	
	2 CS^{y}	6.92	7.11	8.17	8.11	7.88	
	0 CS^{z}	6.67	7.44	7.66	7.69	7.45	
C	7 CS^{x}	6.43	6.32	6.34	6.26	6.21	
	2 CS^{y}	5.40	6.43	6.05	7.01	7.05	
	0 CS^{z}	5.48	6.34	6.33	6.66	6.76	
L	7 CS^{x}	4.33	4.92	5.24	5.35	5.01	
	2 CS^{y}	4.64	4.61	5.76	5.81	5.55	
	0 CS^{z}	4.33	5.42	5.31	5.63	6.07	
	SEM	0.31	0.30	0.30	0.29	0.36	
Main effects[4]							Overall effects
	T	NS	NS	NS	* y≥z≥x	* y≥z>x	* y≥z>x
	L	*** H>C>L	*** H>C>L	*** H>C>L	*** H>C>L	*** H>C>L	*** H>C>L
	I	-	-	-	-	-	*** +5≥+7≥+3>+1>-1
	T x L	NS	NS	NS	NS	NS	NS
	T x I	-	-	-	-	-	NS
	L x I	-	-	-	-	-	NS
	T x L x I	-	-	-	-	-	NS

[a,b]Means within time point of immunization and line group with no common superscript differ significantly ($P<0.05$).

[1] Titers were expressed as the 2log values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of two-fold diluted standard positive plasma present on every microtiter plate. Values are Least Square means of the complete experimental period.

[2]Ten hens per group (duration of CS, line, time point of immunization).

[3]7CS = birds subjected to CS (10^{o}C) for 7 d 2 CS = birds subjected to CS (10^{o}C) for 2 d. 0 CS = birds were kept at 22 ^{o}C continuously (control).

[4]T = effect of duration of CS, L = line effect, I = effect of time point of immunization.

*<0.05, **<0.01, ***<0.001.

Experiment 2. An 'early' *in vitro* lymphocyte proliferation to ConA was determined in blood samples collected at 10 days after immunization with KLH, i.e. days +9, +12, +13, +15, and +16 after CS treatments. A 'late' *in vitro* lymphocyte proliferation to ConA and KLH were determined in blood samples collected at day +20, +22, +24, +26, and +28 after immunization with KLH i.e. at day +28 post CS.

Early In Vitro Lymphocyte proliferation to ConA. There was a significant combined effect of duration of CS, line of bird and time point of immunization on early *in vitro* lymphocyte proliferation to ConA (Table 3); hence results for this parameter are presented per time point of immunization after CS.

An early *in vitro* lymphocyte proliferation to ConA was not significantly affected in birds subjected to any of the three duration of CS and immunized at -1, +1 or +3 day after CS by duration of CS, line, or interactions among CS and line. A treatment by line interaction was found for birds that had been cold stressed and immunized at +5 and +7 day after CS (Table 3). At these time points, neither 7CS nor 2CS significantly affected H line birds when compared with the respective control group. Whereas a suppression effect was found in the C line birds in response to both 2CS and 7CS, and an enhancing effect was found in the L birds in response to 2CS.

Late In vitro Lymphocyte proliferation to ConA. In general, *in vitro* lymphocyte proliferation in the presence of ConA was significantly affected by duration of CS. Birds subjected to 7CS showed highest lymphocyte proliferation followed by 2CS and 0CS (Table 4). There was a significant line effect. Highest proliferation was found in the L line birds followed by the C and the H line birds (Table 4). There was a significant effect of time point of immunization relative to CS on lymphocyte proliferation to ConA. There was a significant combined effect of duration of CS, line and the time point of immunization on lymphocyte proliferation to ConA (Table 4).

From day 25 after immunization, 2CS negatively affected lymphocyte proliferation, whereas 7CS enhanced proliferation in all lines. At 21 days after immunization with KLH, 2CS also enhanced lymphocyte proliferation in the C line. Only at 25 days after immunization a line by treatment effect was found, i.e. both 2 CS and 7CS enhanced lymphocyte proliferation in the L line.

Table 3. Stimulation indices of in vitro lymphocyte proliferation to concanavalin A (ConA[1]) of whole blood at d 10 (d 9 to 11) after immunization from high (H), control (C) and low (L) line hens[2] subjected to short or long duration of cold stress (CS) vs. the control group and immunized with keyhole limpet haemocyanin (KLH) at 1 of 5 time points after CS in experiment 2

		Time point of immunization after CS					
		-1	+1	+3	+5	+7	
		Days after CS					
Line	Treatment[3]	9	12	13	15	16	
H	7CS	111.1	174.3	82.1	52.2[a]	119.7	
	2CS	104.1	91.1	60.9	101.9[b]	145.9	
	0CS	68.2	109.0	74.5	79.0[ab]	157.2	
C	7CS	79.2	91.6	54.6	22.7[a]	88.2[a]	
	2CS	67.3	87.4	50.8	62.3[ab]	87.4[a]	
	0CS	60.5	96.6	63.7	94.0[b]	170.7[b]	
L	7CS	67.1	88.5	59.1	118.0[a]	152.3[ab]	
	2CS	86.4	88.8	85.3	65.3[b]	215.3[a]	
	0CS	61.1	115.0	58.9	99.9[ab]	101.8[b]	
	SEM	20.4	21.0	16.0	17.4	26.6	
Main effects[4]							Overall Effects
	T	NS	NS	NS	NS	NS	NS
	L	NS	NS	NS	NS	NS	**
							H≥L>C
	I	-	-	-	-	-	***
							+7>+1>-1≥+5≥+3
	T x L	NS	NS	NS	*	**	NS
	T x I	-	-	-	-	-	NS
	L x I	-	-	-	-	-	NS
	T x L x I	-	-	-	-	-	**

[ab]Means within time point and line group with no common superscript differ significantly ($P< 0.05$).
[1]Least square means of the complete experimental period.
[2]10 hens per group (Treatment, line).
[3]7CS = birds subjected to CS (10°C) for 7 d. 2 CS = birds subjected to CS (10°C) for 2 d. 0 CS = birds were kept at 22 °C continuously (control).
[4]T = effect of duration of CS, L = line effect, I = effect of time point of immunization.
*P<0.05, **P<0.01, ***P<0.001.

In Vitro Lymphocyte proliferation to KLH

Experiment 1. SI to KLH was affected by a line by treatment interaction (Table 1). There was a significant effect of CS applied at all three time points prior to immunization on the SI of *in vitro* lymphocyte proliferation to KLH. No line effect was found. Significantly enhanced SI to KLH was found in the H and the C line birds subjected to CS at 1 d.p.i. and 8 d.p.i. when compared with their respective control groups. The L line birds subjected to CS at 8 d.p.i showed significantly enhanced SI when compared with the respective control group.

Experiment 2. There was significant combined effect of duration of CS, line and immunization (Table 4). Therefore, the data for this parameter is discussed per time point after immunization. A line effect on lymphocyte proliferation to KLH was only found at 23 days after immunization in birds immunized at +5 days after CS. The H line birds from that group had higher lymphocyte proliferation to KLH (Table 4). Effects of CS on *in vitro* lymphocyte proliferation to KLH were found only at 21, 23 and 29 days after immunization in the groups immunized at -1, +5 and + 7 days after CS. Enhanced lymphocyte proliferation was seen at 29 days after immunization in both H and L line birds subjected to 2CS and at 21 days after immunization in birds subjected to 7CS. Whereas, suppressed lymphocyte proliferation to KLH was seen at 23 days after immunization in H line birds subjected CS and immunized at +5 days after CS.

Reactive oxygen intermediates production (ROI)

Experiment 1. ROI production was affected by a line by treatment interaction (Table 1). There was a significant effect of time of CS before immunization with KLH on ROI production, and also a significant line effect. ROI production was significantly higher in the C line than in the other two lines. The H line birds subjected to CS at 8 d.p.i. with KLH showed significantly enhanced ROI production when compared with the respective 0CS group of the same line. In the C line birds significantly suppressed ROI production was found when subjected to CS at 3 d.p.i with KLH when compared with the 0CS group of the same line. Significantly enhanced ROI production was found in the L line birds subjected to CS at 1 d.p.i. with KLH when compared with the control group of the same line.

Experiment 2. There was significant overall effect of duration of CS on ROI production. Birds subjected to 0CS showed the highest ROI production followed by 7CS and 2CS

Table 4. Stimulation indices of *in vitro* lymphocyte proliferation to ConA of whole blood at day 28 after cold stress from high (H), control (C) and low (L) line hens[2] subjected to either 2, 7 or 0 CS and immunized with KLH at one of the five different time points after cold stress

Line	Treatment[3]	Time point of immunization after cold stress: -1 / Days after immunization: 29		+1 / 27		+3 / 25		+5 / 23		+7 / 21		
		ConA	KLH	ConA	KLH	ConA	KLH	ConA	KLH	ConA	KLH	
H	7 CS^{x}	81.8^{a}	0.66^{a}	115.4^{a}	0.88	113.3^{a}	0.84	63.2	0.71^{a}	94.4	0.86^{a}	
	2 CS^{y}	28.3^{b}	1.01^{b}	29.6^{b}	0.94	16.4^{b}	0.89	71.7	0.80^{a}	61.1	0.71^{a,b}	
	0 CS^{z}	65.3^{a}	0.54^{a}	51.6^{b}	0.72	58.4^{b}	0.86	38.4	1.32^{b}	49.7	0.64^{b}	
C	7 CS^{x}	103.6^{a}	0.75	97.3^{a}	0.74	96.6^{a}	0.86	98.7	0.59	91.4^{a,b}	0.78	
	2 CS^{y}	24.2^{b}	0.93	23.0^{b}	0.92	42.2^{b}	0.87	64.8	0.73	93.2^{a}	0.72	
	0 CS^{z}	92.8^{a}	0.76	72.9^{a}	0.82	75.1^{a,b}	0.93	94.0	0.76	44.4^{b}	0.64	
L	7 CS^{x}	155.0^{a}	0.75^{a}	77.7^{a}	0.73	120.2^{a}	0.96	98.1	0.85	130.7	0.90^{a}	
	2 CS^{y}	33.6^{b}	1.08^{b}	16.7^{b}	0.82	107.5^{a}	0.89	60.0	0.59	122.0	0.62^{b}	
	0 CS^{z}	84.0^{c}	0.70^{a}	68.9^{a}	0.92	59.2^{b}	0.97	47.3	0.69	93.4	0.67^{b}	
	SEM	12.9	0.09	13.2	0.09	15.2	0.10	14.1	0.10	17.1	0.07	
Main effects[4]												Overall effects
												ConA / KLH
	T	*** x>z>y	*** y>x≥z	*** x>z>y	NS	*** x>z≥y	NS	NS	* z≥x≥y	* x≥y>z	** x>y≥z	*** x>y>z / NS
	L	* L≥C≥H	NS	NS	NS	* L≥C≥H	NS	NS	** H>L≥C	** L≥C≥H	NS	*** L≥C>H / NS
	I	-	-	-	-	-	-	-	-	-	-	**[5] / **[6]
	T x L	NS	NS	NS	NS	*	NS	NS	**	NS	NS	NS / NS
	T x I	-	-	-	-	-	-	-	-	-	-	*** / ***
	L x I	-	-	-	-	-	-	-	-	-	-	** / *
	T x L x I	-	-	-	-	-	-	-	-	-	-	* / *

[a-c]Means within time, parameter, and line group with no common superscript differ significantly ($P< 0.05$). [1]Least square means of the complete experimental period. [2]Ten hens per group (treatment, line). [3]Birds subjected to CS (10°C) for 7 d, 2 d or 0 d. [4]T = effect of duration of CS, L = line effect, I = effect of time of immunization. [5]+7≥+3≥-1≥+5≥+1, [6]+3≥+1≥-1≥+5>+7, *P<0.05, ** P <0.01, *** P <0.001.

(Table 5). There was significant line effect, the L line birds had highest ROI production followed by the C and the H line birds (Table 5). There was also a significant effect of time point of immunization on ROI production. For ROI production, the later the time point of immunization relative to CS the higher the ROI production (Table 5). There was a significant combined effect of duration of CS, line and time point of immunization on ROI production (Table 5). Both enhancement and suppression of ROI production was found in all three lines at all time points, depending, however, on line (line by treatment interactions). In general, H line birds were sensitive to CS at almost all time points after CS treatments, C line birds only at 6 days after CS, and L line birds only at 10 and 12 days after CS treatments.

Body weight (gain)

Experiment 1. There was no significant effect of CS applied at all three time points prior to immunization, line effects, or treatment by line interactions on body weight gain (data not shown).

Experiment 2. The time point of KLH immunization did not significantly affect body weight gain. Therefore, this treatment factor was left out of the analysis. Birds were analyzed according to the duration of CS or no CS. Subjecting chicks to CS either for 2 days or for 7 days resulted in significantly reduced body weight gain (Table 6). Especially 7CS suppressed body weight gain. The H line birds gained significantly ($P< 0.05$) higher body weight than the C line and the L line birds. The CS by line interaction significantly affected body weight gain. The L line birds subjected to 7CS gained the least body weight followed by the C and the H line birds subjected to the same treatment.

4.5 Discussion

In the present study we evaluated the effect of duration of CS on various components of the immune system of poultry. Special emphasis was given for both short term and long term effects of duration of CS. We used lines divergently selected for antibody responses to study a possible trade off between investment in different immune system components, thermoregulation and body weight (gain), and to find indications of different gene expression under situations of simultaneous stressors like cold, growth, restricted feeding and activation of the immune system. The main differences between the current two experiments were

Table 5. Reactive oxygen intermediates (ROI) production[1] during zymosan A stimulation in the whole blood samples sampled on day +7 after immunization with KLH from high (H), control (C) and low (L) line hens[2] subjected to either short or long duration of cold stress (CS) and immunized with KLH at one of the five different time points after cold stress in experiment 2

		Time point of immunization after cold stress					
		-1	+1	+3	+5	+7	
		Days after cold stress					
Line[3]	Treatment[4]	6	8	10	12	14	
H	7 CS^{x}	0.47^{a}	0.34	0.42^{a,b}	0.43^{a}	0.43^{a}	
	2 CS^{y}	0.24^{b}	0.36	0.33^{a}	0.58^{b}	0.64^{b}	
	0 CS^{z}	0.49^{a}	0.42	0.51^{b}	0.39^{a}	0.77^{b}	
C	7 CS^{x}	0.74^{a}	0.49	0.49	0.43	0.50	
	2 CS^{y}	0.55^{b}	0.61	0.41	0.46	0.56	
	0 CS^{z}	0.39^{c}	0.50	0.51	0.44	0.52	
L	7 CS^{x}	0.49	0.48	0.62^{a}	0.62^{a}	0.75	
	2 CS^{y}	0.42	0.51	0.32^{b}	0.54^{a,b}	0.57	
	0 CS^{z}	0.50	0.61	0.71^{a}	0.45^{b}	0.58	
	SEM	0.04	0.05	0.06	0.05	0.06	
Main effects[5]							Overall effect
	T	***	NS	***	*	NS	*
		x>z>y		z≥x>y	y≥x≥z		z≥x>y
	L	***	***	*	*	NS	***
		C>L>H	C=L>H	L≥C≥H	L>H≥C		L>C>H
	I	-	-	-	-	-	***
							+7>+5≥+3≥+1≥-1
	T x L	***	NS	NS	*	**	***
	T x I	-	-	-	-	-	***
	L x I	-	-	-	-	-	***
	T x L x I	-	-	-	-	-	***

[a-c]Means within time, and line group with no common superscript differ significantly ($P< 0.05$).
[1]Least square means of the complete experimental period.
[2]Ten hens per group (treatment, line).
[3] 7CS = birds subjected to CS (10^{o}C) for 7 d. 2 CS = birds subjected to CS (10^{o}C) for 2 d. 0 CS = birds were kept at 22^{o}C continuously (control).
[4]T = effect of duration of CS, L = line effect, I = effect of time point of immunization.
*P<0.05, **P<0.01, ***P<0.001.

a) there was an age difference between treatment groups when they were subjected to CS in experiment 1, but all the treatment groups were immunized with KLH at the same age. b) There was no age difference between treatment groups when they were subjected to CS in experiment 2, but there was an age difference between treatment groups when they were immunized with KLH. In the latter experiment, introduction of parallel control groups eliminated age effects of immunization between treatment groups and the control groups. Immune parameters studied were indicative of the specific humoral (antibodies directed to KLH) and cellular (lymphocyte proliferation *in vitro* to ConA and KLH) immune response, and the innate immune system (ROI production by blood monocytes). Reports on the effects of CS on immune parameters of birds are inconsistent. Both enhanced [10], or decreased [11, 14] antibody responses after exposure to CS were reported, whereas Regnier and Kelley [12] found little effect of acute CS on antibody responses. Recently we also found no effects of various duration of CS on specific antibody titers in chicks when immunized at one time point during CS [81]. Cold stress suppressed cellular immune responses in chickens [6]. In two independent studies we previously found evidence that a cold environment enhanced cellular immune responses in chickens [1, 81]. In the former study, we found, however, a differential effect of different duration of CS on the cellular immune component. We therefore predicted that the effect of CS on immune responses may depend on the time point after the stressor at which immune responses are determined, next to the duration of CS.

The first objective of the present studies was to determine the effect(s) of duration of CS and time points of subjecting birds to CS prior to activation of the immune system with a model antigen (KLH) on specific and innate immune responses of selection lines. The results of the current two experiments demonstrate that the duration of cold treatment hardly affected acquired (KLH, Tables 1 and 2) humoral immunity of the selected chicken lines. Some suppressive (7 CS) or enhancing (2 CS) effects on specific antibody responses to KLH were found when birds were immunized 5 to 7 days after the CS treatments. We used genetically selected birds. With respect to humoral immune responses after CS no line by treatment interactions were found. This suggests that divergent genetic selection for primary antibody responses may have resulted in a fixation of genes, leaving little variation to respond differently to either short or long CS treatments in H and L line birds [81].

Table 6. Average BW gain[1] from high (H), control (C) and low (L) line hens[2] subjected to cold stress (CS) for 1 of 3 durations in experiment 2

Line	Treatment[3]	BW gain (g)
H	7 CS^{x}	53.88^{c}
	2 CS^{y}	77.66^{b}
	0 CS^{z}	93.30^{a}
C	7 CS^{x}	41.78^{c}
	2 CS^{y}	72.38^{b}
	0 CS^{z}	88.84^{a}
L	7 CS^{x}	33.48^{c}
	2 CS^{y}	68.78^{b}
	0 CS^{z}	90.96^{a}
	SEM	2.23
Main effects[4]		
	Treatment	*** z>y>x
	Line	*** H>C≥L
	Treatment x Line	***

[a-c]Means within line group with no common superscript differ significantly ($P< 0.05$).
[1]The BW on 35 d of age (BW after CS) minus BW on 28 d of age (BW before CS).
[3]Ten hens per group (treatment, line). [3]0CS = birds were kept in climate chamber at 22 ^{o}C continuously (control); 2 CS = birds subjected to CS for 48 hours before immunization; 7CS = birds subjected to CS for 7 d before immunization. [4]T = effect of duration of CS, L = line effect. ***$P<0.001$.

Cold stress of chickens is associated with depressed cellular immunity [6]. In an earlier study, we however, found enhancing effects of CS on lymphocyte proliferation to ConA when determined at +32 days after CS and immunization [81]. In order to confirm that the enhanced mitogen responses to ConA were the result of a stress response, and not the result of a rebound response to an earlier suppressed mitogen response to ConA during or shortly after CS, we determined both an 'early' and a 'late' mitogen response after CS to ConA in the current study. The early mitogen responses (Table 3) suggest that CS can either enhance or suppress mitogen responses to ConA depending on the chicken line, but these effects were noticed starting from 15 days after CS. Suppressive effects were almost consistent in the C

line birds, but in the L and the H line birds enhancement effects were found. A 'late' lymphocyte proliferation to ConA and KLH were determined as measures of specific adaptive cellular immunity at day +28 after CS. Day 28 after immunization was chosen, since mean KLH-specific lymphocyte proliferation of the current lines is highest between day 28 and day 35 after immunization with KLH. In general, but not always, 7CS enhanced responses to ConA in all three lines, and 2CS suppressed mitogen responses in all three lines (Table 4). Also depending on the time point after immunization, specific lymphocyte proliferation to KLH was affected by CS treatments, the L birds showing enhanced, and the H line enhanced or suppressed specific cellular immunity to KLH. When CS treatment effects were significant, 7CS enhanced lymphocyte proliferation to KLH at an earlier time point after immunization than 2CS, which enhanced antigen-specific lymphocyte proliferation at a later time point. This suggests a dose dependent effect of CS on the time that cellular immunity is affected, or alternatively may indicate different sensitivity of different T-cell populations to CS in time.

The mechanisms underlying the enhancement of lymphocyte proliferation at a considerable duration of time after CS are unknown. Cold stress induced lower corticosterone levels in the plasma of the present chickens (data unpublished). It is tempting to speculate about a non-antigen-specific generalized stress response to cold, which may resemble the non-antigen specific general fat deposition in chickens immunized with model antigens [63]. As was true for antibody responses we found no line by treatment interactions for the effects of CS on cellular immunity. In addition, we found a significant ($P < 0.001$) positive correlation between early and late mitogen responses to ConA (data not shown). This suggests that 1. Enhanced or suppressed mitogen responses after CS are not a rebound effect of an earlier suppression or enhancement caused by the CS, and 2. Immune responsiveness depends on the genetic background of the individual or breed. The absence of line by treatment interactions for the humoral and cellular immune parameters studied, and the different effects of CS on cellular versus antibody responses indicate that type and magnitude of specific immune responses depend on the genotype, whereas the environment influences the magnitude or kinetics of immune responses as we proposed earlier [1].

A significant enhancement, suppression, or no effect of short duration of CS on ROI was found depending on the time point of measurement, the time points of CS prior to immunization and the genotype. Also ROI was characterized by treatment by line

interactions. Though these results are difficult to explain they suggest that ROI is much more sensitive for environmental stressors, and less restricted to the genotype as proposed for the specific immune system. A comparison of the results of the present study with a previous study [81], suggests that the time point of measurement of ROI after CS is more important than the intensity of the CS itself.

Our second objective was to measure the importance of the time points of CS prior to activation of the immune system, in combination with the duration of the stress. For specific immune responses to KLH, treatment (CS) effects appeared mostly related with the time point of stress prior to immunization, whereas the intensity (either 2 or 7CS) of the stressor appeared to be of less importance. The time point of measuring non-specific immune responses (mitogen responses and ROI production) after stress appeared more important than the time after immunization. In the present studies, the challenge (intensity and duration) of the stressor may, however, not have been sufficient enough to detect consistent effects on immune parameters in the present chicken lines. Thus, future studies should focus on the effects of a prolonged period of simultaneous stressors like cold and restricted feeding on overall immunity, which might resemble natural conditions such as winter.

The third objective of the present study was to study a possible energy-related trade-off between different components within the immune system, and between the immune system and a life trait such as growth. There was no clear trade-off between cellular and humoral immune components. Our data show that CS resulted in a substantial decrease in body mass (Table 6). Especially H line birds showed significantly lower mitogen responses to ConA but gained more body weight than the other two lines. In the present experiments birds had a limited supply of feed. Hence, birds could not compensate by increasing food intake for the expected extra energy requirement for thermo-regulation. Previously we found no energy requirement of the humoral immune response in poultry [63]. On the other hand, systemic acute phase response [64], and perhaps cellular immune responses are energy demanding. We calculated a significant negative correlation for body weight gain and the late *in vitro* mitogen response (data not shown), suggesting a form of trade-off between energy demanding cellular immune responses and body weight gain.

In conclusion, effects of duration and time points of application of CS prior to activation of the immune system under situations of restricted feeding and growth are not consistent. A

longer duration of CS generally but not always enhanced CMI, but humoral immunity was hardly affected. Effects on the specific immune response appeared more related with the status of activation of the immune system, whereas parameters of innate immunity were much more sensitive to the intensity and duration of the stressor and the time point of measuring these parameters after stress. The duration of CS in the present studies may not have been sufficient to measure consistent effects of CS on overall immune responsiveness. Mere application of cold exposure does not guarantee that thermal stress is strong enough to induce a trade-off between immune function and physiological adaptations to increased heat loss.

Hence, we propose studies with prolonged CS on selection lines to better mimic long-term effect under more natural conditions on immunity and to identify the proposed trade-off between immune system and various other life traits, such as thermoregulation and growth, with special emphasis on cellular immune parameters. Furthermore, our data indicate that quantifying one component of the immune system, either humoral or cell-mediated immune response may not be sufficient to estimate overall immunocompetence (health status) of an animal under stress.

Chapter 5

Severe feed restriction enhances innate immunity but suppresses cellular immunity in chicken lines divergently selected for antibody responses

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5.1 Abstract

The main aim of this study was to investigate the effects of different levels of feed restriction (FR) on immune responses of chicken lines divergently selected for high (H) and low (L) antibody responses to SRBC. We hypothesized that the severe feed restriction suppresses immune responses and the level of immune suppression differs between birds with different genetic background. Therefore, we tested antibody responses, blood lymphocyte proliferative response and reactive oxygen intermediates (ROI) production by Zymosan-A stimulated blood cells of chicken lines maintained on three levels of FR. The H line birds had higher antibody responses, higher ROI production, and lower corticosterone (CORT) levels when compared with the L line birds. FR induced no effect on specific antibody responses to either a Th-1 (*Mycobacterium butyricum*) or a Th-2 (KLH) type antigen. FR birds showed a marked reduction in natural antibodies binding lipoteichoic acid (LTA), *in vitro* lymphocyte proliferation in response to stimulation with concanavalin A, body weight (BW) gain, and lymphoid organ relative weights when compared with the birds fed *ad libitum*. However, FR birds showed a markedly enhanced ROI production, and plasma CORT levels when compared with the birds fed *ad libitum*. The enhanced ROI production and suppressed lymphocyte proliferation coinciding with enhanced plasma CORT levels suggest stress-mediated immunomodulating effects of FR. A significant treatment by line interaction was found for ROI production; the increase of ROI production was higher in the H line than in the L line under severe FR condition. With respect to CORT levels; the increase in CORT levels was larger in the L line than in the H line under severe FR condition. Furthermore, the L line gained more BW than the H line under *ad libitum* condition. Finally, under severe FR, relative spleen weight was smaller in the L line than in the H line. The present findings suggest genetic differences affecting physiological and immunological responses under FR conditions.

5.2 Introduction

Recently we studied the consequences of an increased energy expenditure to maintain body temperature on immune responsiveness of poultry by subjecting growing birds to cold stress for various durations prior to immunization, and comparing their immune responses with non cold-stressed control groups [81, 104]. Furthermore, to identify possible genetic differences chicken lines divergently selected for 22 generations for high and low antibody responses to SRBC were used. The significance of energy trade-offs (priorities) between physiological processes and thus the consequences of various forms of energy demanding stress responses can only be measured under conditions where energy supplies are limited. Otherwise, increased rates of energy acquisition rather than its reallocation from other essential components of energy budget [26] may satisfy the extra costs incurred by thermoregulation, growth and immune challenge. For this reason in our previous studies, we burdened growing birds from both lines with immune stress as well as cold stress under restricted feeding conditions [81, 104].

Some studies have shown that feed restriction alone leads to stress resulting from hunger and frustration [32], which may result in impaired health and welfare. As proposed earlier by Gursoy et al. [105], exposure to multiple stressors at a time may have synergistic, additive or adaptive effects on the immune parameters measured [81, 104]. Many investigators have examined the effect of feed restriction alone on various parts of the immune system, such as hematological values [32, 33], macrophage function [34, 35], antibody responses [30, 36-39], cell-mediated immunity [30, 36, 39-41] and corticosterone levels [32, 42-44]. However, there are only few studies in which different components of the immune system have been studied simultaneously in a single experiment. Moreover, it is well documented that immunocompetence and growth are affected by genetic background, nutrition and environment [38], but the genetic component has not been considered in the studies mentioned above.

We studied the effect of feed restriction on immune responses to a Th-1 and a Th-2 antigen, BW gain and CORT levels of chicken lines divergently selected for antibody responses. Incorporation of two levels of feed restriction (moderate, the level of feed restriction used in the previously reported studies, and severe, higher level of feed restriction than the level used in the previous studies) in the present experiment should provide an

opportunity to investigate the change in the competence of different components of the immune system; e.g., innate, humoral and cell-mediated, along a gradient in availability of resources and the duration of FR. Moreover, in the present experiment important physiological parameters were monitored at weekly interval for a period of 8 weeks to measure adaptive responses to the limited resource conditions. Since the current chicken lines selected for antibody responses differ in almost every aspect of specific and non-specific immune responses and also in body weight, these birds offered the opportunity to study possible trade-offs between genetically based investment in different components of the immune system and another physiological priority such as growth.

5.3 Materials and methods

Chickens, housing and feed

One hundred and twenty 23-d-old ISA Warren Brown Medium Heavy layer chickens from two lines were used in this study. These two lines were divergently selected for 22 generations for either the high (H line) or the low (L line) primary antibody responses at d 5 after intramuscular (i.m.) immunization with SRBC at 35 d of age [57]. They were vaccinated for Marek's disease and infectious bronchitis at hatch and infectious bursal disease on d 15 of age. Birds were kept according to routine procedures for layers in brooder cages. The light regimen was 14 h L (04.00 till 18.00):10 h D. They were fed with a commercial diet (200 g kg^{-1} CP, 2600 kcal kg^{-1} ME), and had free access to water throughout the experiment. From the day of hatch, until 23 d of age birds had *ad libitum* access to feed. The feeding regime from 23 d of age is described in the experimental design section.

Experimental design

Effects of feed restriction on immune responses in two chicken lines were studied using 3 x 2 x 2 x 10 factorial design. Factors were feeding levels, lines of chicken, antigens used for immunization and number of birds per experimental unit, respectively. Each experimental unit consisting of 10 birds (/treatment/line/immunization) were housed in a brooder cage during the study. At 23 d of age, 2 experimental units of each H and the L line were randomly assigned to one of the three feeding treatments. Control groups received feed *ad libitum* (175-

190% of MEm), and birds assigned to restricted regimes were fed 80% (160% of MEm), or 40% (120% of MEm), respectively, of the feed consumed by the *ad libitum* fed group. Daily feed intake of the control groups was determined by subtracting the quantity of uneaten feed in the feeding tray at 8:00 AM from the quantity of feed supplied in the feed tray the day before at 8:00 AM. Mean feed intake for the H line birds fed *ad libitum*, and 20% and 60% restricted were 31.5, 24.3 and 16 g/d during week 1; 47, 35.5, 20.5 g/d for week 3; and 60.5, 48, 25.5 g/d for week 5, respectively. Mean feed intake for the L line birds fed *ad libitum*, and 20% and 60% restricted were 35, 24.3 and 17.5 g/d during week 1; 50, 35.5, 20.5 g/d for week 3; 68.5, 49, 25.5 g/d for week 5, respectively.

At 37 d of age, birds in one experimental unit of each line, kept at each feeding level (10 birds each per line per feeding group) were immunized s.c. with 1 mg keyhole limpet hemocyanin (KLH, Cal Biochem – Novabiochem Co., San Diego, CA) dissolved in 1 mL PBS / bird. Whereas, birds in another experimental unit of each line, kept at each feeding level were immunized with 1 mg heat killed *Mycobacterium butyricum* (Difco, Detroit, MI) dissolved in 1 mL PBS / bird. KLH and *M. butyricum* are the antigens which chickens do not encounter during their lifetime. Thus, they represent novel antigens which are suitable to measure primary immune responses in chickens. In mammals, KLH immunization results in Th-2 type of immune responses [106]. On the other hand, immunization with *M. butyricum* results in Th-1 type of immune response [107]. One mL heparinized blood samples were collected from wing vein at d 0 (d of immunization), and subsequently at 1, 7, 14, 21 and 28 d after immunization. Blood samples were collected within 3 min of catching birds from their cages. Blood samples collected at 7, 14, 21 and 28 d after immunization were used to measure reactive oxygen intermediate production, and lymphocyte proliferation to concanavalin (Con A, Sigma Chemical Co., St. Louis, MO). Specific antibody titers to KLH and *M. butyricum*, and natural antibody titers to *Escherichia coli* lipopolysaccharide (LPS, Sigma Chemical Co., St. Louis, MO), and *Staphylococcus aureus* lipoteichoic acid (LTA, Sigma Chemical Co., St. Louis, MO) were determined in plasma collected at all sampling days. Blood samples collected at d 0, 7, 14, 21 and 28 after immunization were used to measure corticosterone levels. Individual BW were recorded weekly.

Assays

ELISA. Antibodies binding to KLH, *M. butyricum*, LPS or LTA were determined in individual plasma samples by indirect two-step ELISA. Plates coated with 1 μg/mL KLH, or 1 μg/mL *M. butyricum,* or 4 μg/mL LPS, or 4 μg/mL LTA dissolved in carbonate buffer (pH 9.6), and subsequently incubated with serial dilutions of plasma. Binding of antibodies to the antigens was detected using 1:20,000 diluted rabbit anti-chicken IgG_{H+L} labeled with peroxidase ($RACh/IgG_{H+L}/PO$, Nordic, Tilburg, The Netherlands). After washing, freshly prepared substrate solution tetramethylbenzidine in 0.02 *M* sodium citrate buffer (pH 5.5) containing 0.05% H_2O_2 was added, and incubated for 10 min at room temperature. The reaction was stopped by adding 2.5 N H_2SO_4. Extinctions were measured with a Multiskan (Labsystems, Helsinki, Finland) ELISA reader at a wavelength of 450 nm. Titers were expressed as the 2log values of the dilutions that gave extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of standard positive plasma present on every microtiter plate.

In vitro lymphocyte proliferation Assay. *In vitro* lymphocyte proliferation in response to Con A stimulation was measured as described in chapter 2.

Reactive oxygen intermediates production assay. The 'Nitroblue Tetrazolium Assay' (NBT) was used to measure reactive oxygen intermediates (ROI) production by phagocytes in whole blood as described in chapter 2

Corticosterone. Corticosterone levels in plasma samples collected from KLH immunized animals were quantified using a commercial radioimmunoassay kit (IDS, Inc, Boldon, UK) and the concentrations were expressed in ng/mL. This kit was validated for use in chicken plasma by Buyse et al. [100].

S*tatistical analysis*

Differences in titers of plasma antibodies binding KLH, and *M. butyricum* were analyzed by a three-way ANOVA for the effect of feed restriction, line, time, and their interactions by the repeated measurement procedure using a 'bird nested within treatment and line' option. Differences in titers of plasma antibodies binding LPS, LTA and body weight were analyzed

by a four-way ANOVA for the effect of feed restriction, line, immunization, time, and their interactions by the repeated measurement procedure using a 'bird nested within treatment, line and immunization' option. The nested term was used as an error term to test treatment and line effects. A two-way ANOVA was performed to determine differences between treatments and lines and their interaction with respect to lymphocyte proliferation in response to stimulation with Con A, ROI production and CORT levels at each time point. Body weight gain, relative spleen and bursa weight data were analyzed using tow-way ANOVA. Pearson's correlation analysis was performed to measure trade-offs between growth and immune competence and also between different components of immune system. The threshold for significance was $P< 0.05$. All analyses were according to SAS [59].

5.4 Results

Specific antibody responses

The mean specific antibody responses to KLH and *M. butyricum* in birds subjected to FR are shown in Table 1. There was no significant interaction in time between treatment and line. However, significant interactions between treatment and time, and line and time were found. The antibody responses were not affected by level of feed restriction (no significant treatment effect, Table 1), but differed between lines (significant line effect, Table 1). Antibody responses were significantly higher in the H line than the L line. However, interactions between level of feed restriction and line for both KLH and *M. butyricum* were not significant.

Natural antibody levels (NAb)

The mean NAb levels in birds subjected to FR are shown in Table 2. A significant interaction between treatment, line, immunization and time was found for NAb binding LPS but not for LTA. Significant interactions of time with treatment, line and immunization were found for both LPS and LTA binding NAb levels. A significant interaction between treatment, line and immunization was found for NAb binding LPS but not for LTA. FR did not have a significant effect on LPS binding NAb levels. However, birds subjected to FR had significantly less LTA binding NAb levels (significant treatment effect, Table 2). The lines differed significantly in NAb binding both LPS and LTA (significant line effect, Table 2).NAb levels were significantly higher in the H line than the L line birds.

Table 1. Effect of no (ad libitum), moderate (20%) or severe (60%) feed restriction (FR) on average specific antibody titers[1] to keyhole limpet hemocyanin (KLH) and *Mycobacterium butyricum* in hens[2] selected for high (H), and low (L) antibody responses

Line	Treatment	KLH	*M. butyricum*
High	Ad libitum	5.88[3]	6.38
	20% FR	5.58	6.33
	60% FR	5.37	7.06
Low	Ad libitum	3.88	4.22
	20% FR	3.14	4.42
	60% FR	3.17	4.78
	Pooled SEM	0.34	0.37
Effects			
	Treatment (T)	NS	NS
	Line (L)	***	***
		H > L	H > L
	Time (Ti)	***	***
	T*L	NS	NS
	T*Ti	***	**
	L*Ti	***	***
	T*L*Ti	NS	NS

[1]Titers were expressed as the 2log values of the dilutions that gave extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of standard positive plasma present on every microtiter plate.

[2]10 hens per group (treatment, line).

[3]Values are least square means of antibody titers determined in plasma samples collected at d 0, 1, 7, 14, 21 and 28 after immunization. Birds were immunized s.c either with 1mg of KLH or with 1mg of *M. butyricum* in PBS after 2 wk of FR. The data presented in the above table were analyzed by 3-way ANOVA for the effect of FR, line, time and their interactions by repeated measurement procedure using a bird nested within treatment and line option.

*** $P< 0.001$, ** $P< 0.01$, NS=Not significant.

There was a significant effect of the type of antigen used for immunization on NAb levels binding LPS or LTA (significant immunization effect, Table 2). The birds immunized with KLH had significantly higher NAb levels than the birds immunized with *M. butyricum*.

In Vitro Lymphocyte proliferation to Con A

In vitro lymphocyte proliferation in response to stimulation with Con A in birds subjected to FR are shown in Fig. 1. Neither moderate nor severe FR significantly affected *in vitro* lymphocyte proliferation in response to stimulation with Con A at 3 or 4 wk after FR. However, both levels of FR suppressed *in vitro* lymphocyte proliferation in response to stimulation with Con A in both H (Fig. 1A) and L (Fig. 1B) line birds at 5 and 6 wk after FR.

Reactive oxygen intermediates production (ROI)

ROI production in birds subjected to FR is shown in Fig. 2. There was a significant treatment by line interaction. Moderate FR significantly suppressed ROI in the H line birds at 6 wk after FR (Fig. 2A); on the other hand, it significantly enhanced ROI in the L line birds at 3 and 6 wk after FR (Fig. 2B). However, severe FR significantly enhanced ROI production in the H line birds at all time points of measurement (Fig. 2A).

Body weight gain [BWG (g)], relative Spleen and Bursa weights (g / kg)

BWG, relative spleen and bursa weights are shown in Table 3. BWG was significantly affected by the interaction between treatment and line (Table 3). The L line gained significantly more BW under *ad libitum* condition than the H line. The birds subjected to FR gained significantly less BW when compared with the *ad libitum* groups. Moreover, lines did not differ significantly in BWG under FR condition.

Relative spleen weight was also significantly affected by the interaction between treatment and line (Table 3). The relative spleen weights of the H line birds were more affected by FR than the relative spleen weights of the L line birds under FR conditions. There was no significant interaction between treatment and line for relative bursa weight (Table 3). Birds subjected to severe FR had lower relative spleen and bursa weights. The lines also differed significantly for the relative weights of spleen and bursa. The H line birds had higher relative

Table 2. Effect of no (ad libitum), moderate (20%) or severe (60%) feed restriction (FR) on average natural antibody titers[1] to LPS[2] and LTA[3] in hens[4] selected for high (H), and low (L) antibody responses, immunized with either keyhole limpet hemocyanin (KLH) or *Mycobacterium butyricum*

Line	Treatment	LPS Ab		LTA Ab	
		KLH	*M. butyricum*	KLH	*M. butyricum*
H	Ad libitum	6.67[5]	4.71	3.04	2.66
	20% FR	5.47	4.70	2.72	2.31
	60% FR	6.22	4.64	2.56	2.43
L	Ad libitum	4.54	2.67	1.58	1.07
	20% FR	4.79	2.55	1.44	1.20
	60% FR	4.70	2.99	1.02	0.97
	Pooled SEM	0.32		0.18	
Effects					
	Treatment (T)	NS		* ad lib[6] ≥ 20% ≥ 60%	
	Line (L)	*** H > L		*** H > L	
	Immunization (I)	*** KLH > MB		** KLH > MB	
	Time (Ti)	***		***	
	T*L	NS		NS	
	T*I	NS		NS	
	L*I	***		NS	
	T*L*I	***		NS	
	T*Ti	*		**	
	L*Ti	***		***	
	I*Ti	***		***	
	T*L*I*Ti	**		NS	

[1]Titers were expressed as the 2log values of the dilutions that gave extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of standard positive plasma present on every microtiter plate.

[2]*Escherichia coli* lipopolysaccharide (LPS). [4]*Staphylococcus aureus* lipoteichoic acid (LTA).

[4]10 hens per group (treatment, line). [5]Values are least square means of antibody titers determined in plasma samples collected at d 0, 1, 7, 14, 21 and 28 after immunization. Birds were immunized s.c either with 1mg of KLH or with 1mg of *M. butyricum* in PBS after 2 wk of FR. The data presented in the above table were analyzed by 4-way ANOVA for the effect of FR, line, immunization, time and their interactions by repeated measurement procedure using a bird nested within treatment, line and immunization option.

*** $P < 0.001$, ** $P < 0.01$, NS=not significant.

spleen and Bursa weight than the L line birds under both *ad libitum* as well as FR conditions.

Corticosterone levels

Corticosterone levels in birds subjected to FR are shown in Fig. 3. In neither of the two lines, moderate FR did have a significant effect on CORT levels, except for the significant suppressive effect determined in the H line at 6 wk after FR. However, severe FR significantly enhanced CORT levels at 4 and 5 wk after FR in the H line birds (Fig. 3A) and at 3, 4 and 5 wk after FR in the L line birds (Fig. 3B). A treatment by line interaction was significant. The increase in CORT levels was significantly larger in the L line than in the H line birds when subjected to severe FR.

Correlation analysis

Correlation analysis performed using the data of all parameters measured revealed neither a significant positive nor a significant negative relationship between the immune parameters measured in the present study (data not shown).

5.5 Discussion

In the present study effects of FR on various immune parameters and BWG of chicken lines selected for high and low antibody responses against SRBC were studied. In addition to immune parameters, plasma CORT levels were measured to identify possible indirect immunomodulating effects of FR via a stress induced endocrine pathway. Feed restriction is synonymous with terms like calorie restriction and dietary restriction, and has been well studied in rodents. Dietary restriction while avoiding malnutrition can be accomplished by a 20-60% reduction from average unrestricted food intake, including balanced decrease in calories, protein, vitamins and minerals [42, 108, 109]. This regimen results in a limited period of weight loss, after which the animals maintain stable body weight or gradually regain some of the weight originally lost despite continued dietary restriction [40, 108, 110]. In the present experiment, FR did not affect specific antibody titers in response to both KLH and *M. butyricum* immunization. The present finding is in agreement with an earlier report indicating

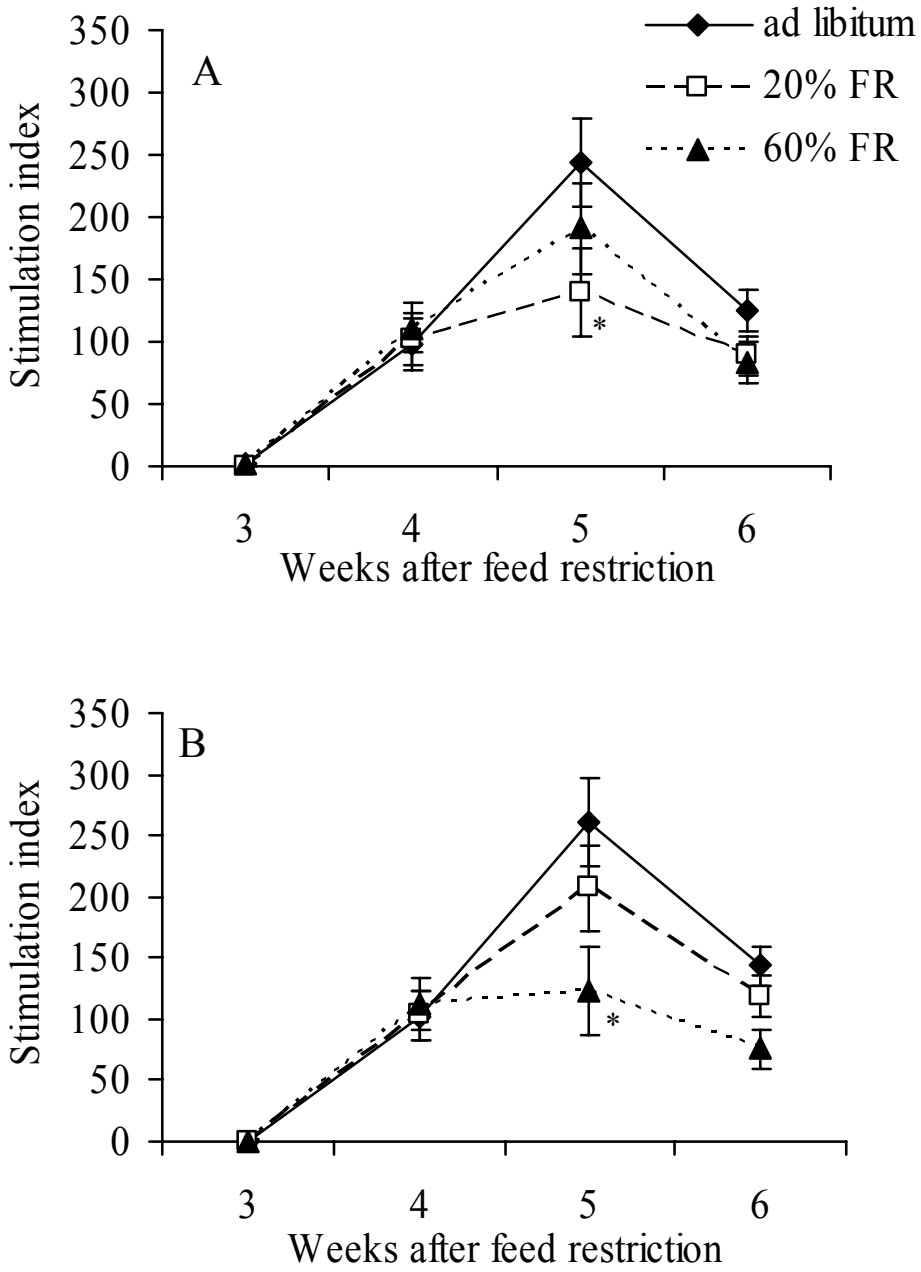

Figure 1. Effect of no (ad libitum, ♦), moderate (20% FR, □) or severe (60% FR, ▲) feed restriction (FR) on stimulation indices of in vitro lymphocyte proliferation in hens selected for high (A) and low (B) antibody responses. In vitro lymphocyte proliferation was measured after stimulating the whole blood samples, collected at 3, 4, 5 and 6 wk after FR, by concanavalin A. Data are means ± S.E (n = 10). Two-way ANOVA was applied, and pair wise comparisons were performed following a significant interaction. Stimulation index = mean cpm of concanavalin A stimulated cultures divided by mean cpm of unstimulated cultures. *Within a line, FR group/s differed significantly when compared with ad libitum group, $P < 0.05$.

that processes leading to specific antibody secretion remain intact over a wide range of nutritional status [111]. However, our finding is in contrast with the previously reported enhanced antibody responses to SRBC in feed restricted broilers [38], and suppressed antibody responses in feed restricted mice and poults to T cell-dependent [30, 37] as well as to T cell- independent antigens [37]. Whether these discrepancies caused by the nature of the antigens used or type of immune response measured remains to be established. Both KLH as an antigen that initiates Th-2 (antibody) responses, and *M. butyricum* as an antigen that initiates Th-1 (inflammation) responses in mammals were studied. Mounting antibody responses to Th-2 antigens was previously shown not to affect metabolism and energy requirements of chickens [63], Similarly, the present results also indicate that the antibody response to a Th-1 antigen may not be a very energy demanding process.

Natural antibodies are part of innate immunity, they facilitate antigen uptake, processing and presentation by B lymphocytes, induce or prevent auto-immune responses, clear lipopolysaccharides, lipoteichoic acid and debris, protect against infection, or they are involved in a hypothetical immunoregulatory idiotype-antiidiotype network [61, 112]. To quantify the natural antibody levels, we chose LPS and LTA as representative antigens of the intestinal micro flora. In the present study levels NAb binding LTA were significantly suppressed by FR. Whether this effect reflects a change in the immune competence of birds due to FR or is an indicator for sensitivity of an individual for an infection remains to be studied. In the present study, enhanced antibody levels to both LPS and LTA were found after immunization with KLH and *M. butyricum,,* which is in agreement with previous report [81].

Severe dietary restriction can often result in a significant suppression of mitogenic responses of lymphocytes *in vitro* [41, 113]. Similar results were found in the present study; however, when birds were feed restricted for more than four weeks. This suggests that birds under prolonged FR conditions cannot keep up their T cell proliferative capacity, which is not contradictory to the idea that indeed the cellular component of the immune system is an energy demanding process [64].

Both moderate and severe FR significantly enhanced some innate immune responses, such as ROI. This finding is in agreement with previously reported enhanced nitric oxide production in murine models upon FR [35]. However, the present finding contradicts the previously reported suppressed phagocytic activity of macrophages in feed restricted broilers

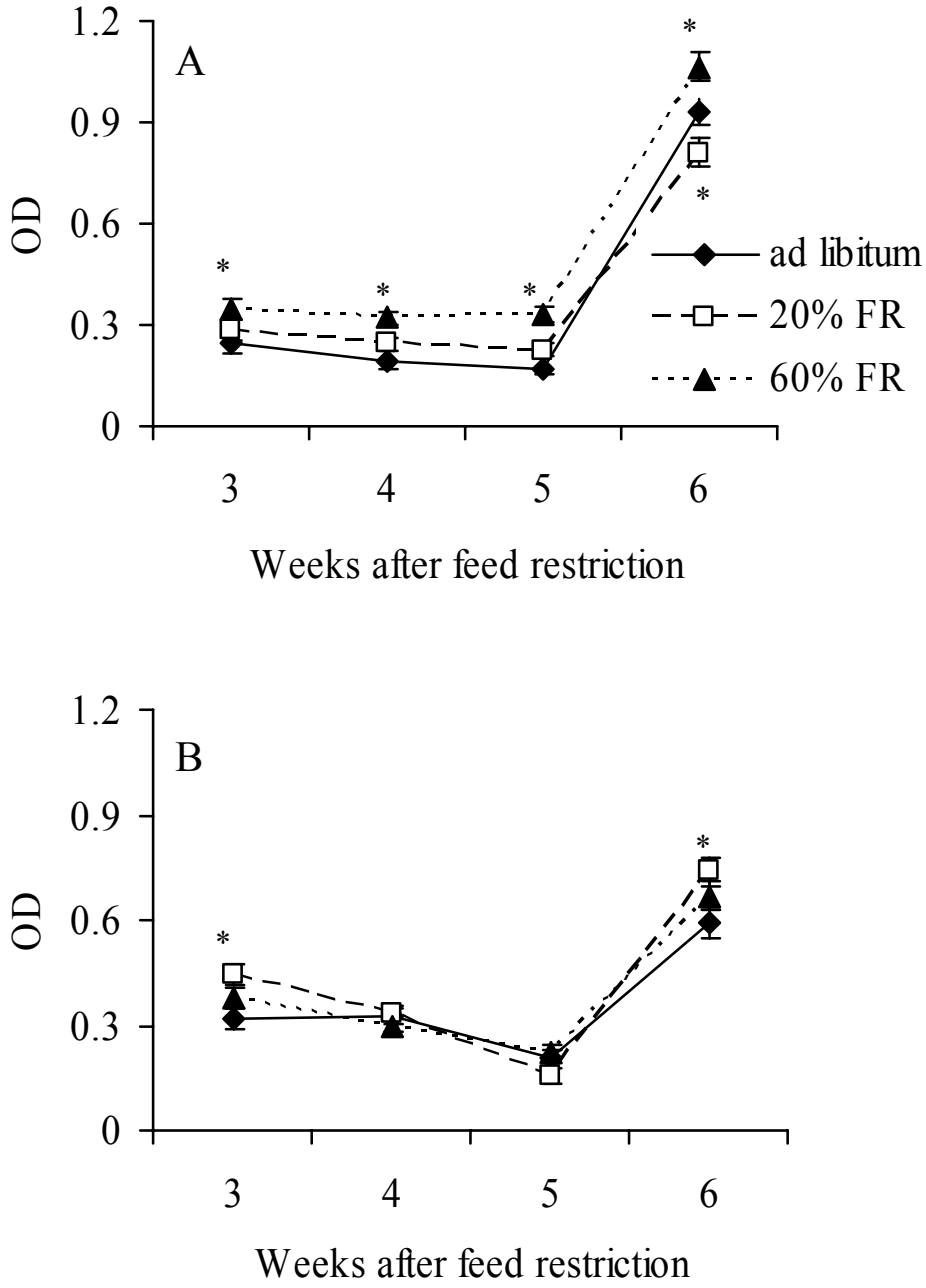

Figure 2. Effect of no (ad libitum, ♦), moderate (20% FR, □) or severe (60% FR, ▲) feed restriction (FR) on innate immunity measured as reactive oxygen intermediates (ROI) production in hens selected for high (A) and low (B) antibody responses. ROI production by peripheral monocytes was measured after stimulating the whole blood samples, collected at 3, 4, 5 and 6 wk after FR, by Zymosan A. Unit of measurement was optical density at 690nm. Data are means ± S.E (n = 10). Two-way ANOVA was applied, and pair wise comparisons were performed following a significant interaction. *Within a line, FR group/s differed significantly when compared with ad libitum group, $P < 0.05$.

Table 3. Effect of no (ad libitum), moderate (20%) or severe (60%) feed restriction (FR) on average BW gain, relative spleen and bursa weight of hens[1] selected for high (H), and low (L) antibody responses

Line	Treatment	BW gain (g) [2]	Spleen weight[3] g / kg BW	Bursa weight[3] g / kg BW
H	ad libitum	384.3[a]	4.10[a]	3.65[ab]
	20% FR	280.5[b]	3.78[a]	4.07[a]
	60% FR	140.6[c]	3.04[b]	3.18[b]
L	ad libitum	416.8[a]	1.56[a]	3.66[ab]
	20% FR	290.6[b]	1.28[ab]	3.25[a]
	60% FR	121.6[c]	1.05[b]	2.27[b]
	Pooled SEM	7.5	0.12	0.22
Effects				
	Treatment (T)	***	***	***
		ad lib[3]> 20% > 60%	ad lib>20% > 60%	ad lib=20% > 60%
	Line (L)	NS	***	**
			H>L	H>L
	T * L	**	*	NS

[a-c]Means with no common superscript differ significantly within a line and the parameter ($P< 0.05$).
[1]10 hens per group (treatment, line).
[2]Values are least square means of BW gain of treatment groups during the experimental period. Body weight gain was calculated before statistical analysis by subtracting the individual BW in the beginning of the experiment (23 d of age) from the individual BW at the end of the experimental period (65 d of age)
[3]Values are least square means of relative spleen / bursa weights of treatment groups measured at the end of the experiment (66 d of age). All the data presented in the above table were analyzed by 2-way ANOVA for the effect of FR, line, and their interactions by repeated measurement procedure using a bird nested within treatment, and line option.
$P< 0.05$, **P<0.01, ***P<0.001, NS=Not significant.

[34]. These apparent contradictions can be attributed to differences in animal models, severity of feed restriction, age of the experimental animal and experimental design. The results of the present study are in agreement with the previously reported fact in human that cell-mediated and nonspecific immunity are more sensitive than humoral immunity to metabolic changes and nutrient deficiencies [114].

As expected, severely FR birds had lower body weight gain, relative spleen weight and relative bursa weight followed by the moderately FR birds when compared with the *ad libitum* fed birds. The line difference with respect to BWG was only seen under *ad libitum* conditions as reported earlier [55], whereas significant line differences in relative spleen weights were seen both under FR and *ad libitum* conditions. There was no significant effect of immunization on BWG of birds. The absence of a significant effect of immunization on BWG is in agreement with the results obtained in other studies [14, 63]. This finding also suggests that the metabolic costs of mounting antibody responses are probably negligible. The significant line by treatment (FR) interaction with respect to BWG and relative spleen weights suggest that the L line birds were more affected by the FR than the H line birds. This suggests that under *ad libitum* conditions the L line birds may spend more energy towards BWG, whereas under FR conditions the same birds may spend more energy towards cellular immune responses.

CORT is the predominant corticosteroid secreted by the domestic fowl adrenal gland [44]. Feed restriction causes higher plasma CORT levels, which in turn suppresses the immune response, possibly through its effects on cytokine production [43]. In the present study significant effects of FR on plasma CORT levels was found only in the birds subjected to severe FR, but not in the birds subjected to moderate FR. Our findings are in agreement with the previously reported increased plasma CORT levels in severely FR chickens when compared with the moderately FR chickens and chickens fed *ad libitum* [115, 116]. Furthermore, the magnitude of enhancement of plasma CORT level was affected by the duration of FR and the line of bird. The present finding contradicts the enhanced CORT production with duration of FR regardless of the genetic background of chickens [44]. This could be attributed to the different experimental methods used to measure the CORT level. The effects of FR on the immune parameters and plasma CORT obtained in the present study suggest that FR partially affects immune responses via the stress mediated endocrine pathway.

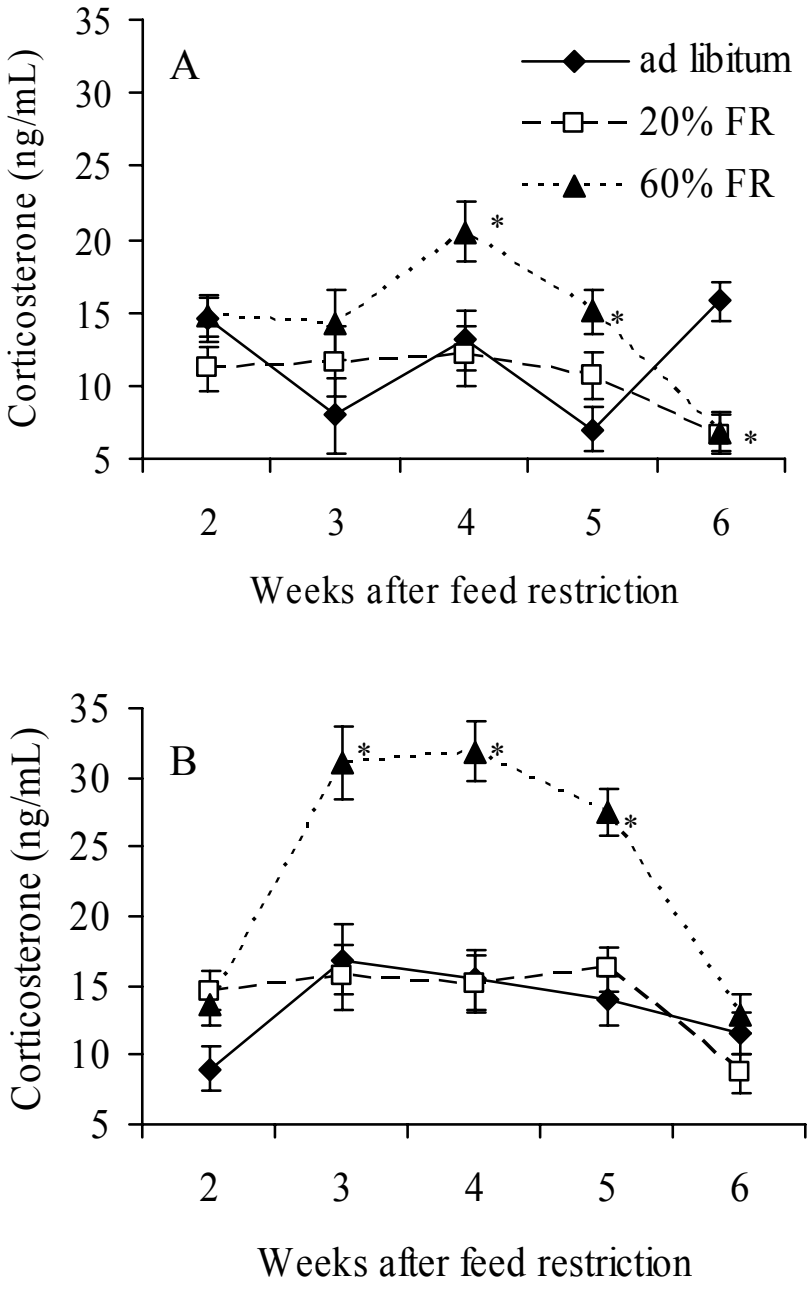

FIGURE 3. Effect of no (ad libitum, ♦), moderate (20% FR, □) or severe (60% FR, ▲) feed restriction (FR) on plasma corticosterone levels in hens selected for high (A) and low (B) antibody responses. Corticosterone levels were measured in plasma samples, collected at 2, 3, 4, 5 and 6 wk after FR. Data are means ± S.E (n = 10). Two-way ANOVA was applied, and pair wise comparisons were performed following a significant interaction. (*Within a line, FR group/s differed significantly when compared with ad libitum group, P <0.05.).

The difference in magnitude of enhancement of CORT level in the H and the L lines suggests that the genetic component greatly influences the effect of FR on adrenocortical function. There were no significant effects of moderate FR on immune and endocrine parameters measured in this study. The present finding indicates that moderate FR did not have synergistic or additive effect on the impact of cold stress measured on the immunocompetence of birds under moderate FR condition [81, 104].

In the present study, even under FR conditions higher antibody (specific as well as natural) levels, higher ROI production and bigger lymphoid organs coincided with lower CORT in the H line. On the other hand, lower antibody levels, lower ROI production and smaller lymphoid organs coincided with higher CORT in the L line. The absence of significant interactions between FR and line on specific antibody and cell mediated immune responses suggests that regardless of genotype, the birds respond similarly to the environmental stressor. However, environmental stress modulates the magnitude of immune and endocrine responses indicating additive relations between genotype and environment, as we found before [1].

Although, we expected a negative effect of FR on immune parameters, we found differential effects. The FR did not have significant effect on specific antibody responses for both Th-1 and Th-2 type of antigen, whereas FR significantly suppressed cell mediated immunity and significantly enhanced innate immunity. This finding suggests that 1) innate and cellular immune components as opposed to humoral immune component are more sensitive to stress and more energy demanding as proposed earlier by Hangalapura et al. [81], Klasing [64] and Lochmiller et al. [41], and 2) even though the innate component of immunity might be energy demanding, it might have priority over the specific component of immunity, as hypothesized by Lochmiller and Deerenberg [56].

Chapter 6

Genetic background determines the effect of cold stress on arms of immune system in domestic fowl *Gallus domesticus*

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6.1 Abstract

Life-history theory predicts a trade-off between immune defense and other vital processes such as thermoregulation, growth and reproduction. We hypothesized that the genetic background of an animal determines the trade-off. To test this hypothesis, we increased the costs of thermoregulation of young female domestic chickens *Gallus domesticus* of lines divergently selected for high (H line) and low (L line) antibody responses to sheep erythrocytes. Restricted feeding was used to limit the ability of chickens to increase energy acquisition by excess feed intake upon cold exposure. We found both positive and negative effects of increased costs of thermoregulation on immune functions, depending on the arm of the immune system and the genetic background. Innate immunity was suppressed, but cellular immunity was enhanced in H line whereas, opposite effects were seen in L line. A specific humoral response was not affected, whereas body weight was reduced equally by cold exposure in both selection lines. The present experimental findings suggest the absence of a direct energetic trade-off between increased costs for thermoregulation and immunocompetence in these chickens. However, the present findings provide an experimental support for trade-offs between the different arms of the immune system. Moreover, the data suggests that the observed trade-off between the arms of the immunity is influenced by the genetic background of an animal.

6.2 Introduction

While it is generally accepted that environmental stressors influence the severity of infectious diseases in birds and mammals, their effects on specific immunological function are relatively unknown [6, 117]. In particular, the influences of cold exposure on immune responses are not clear. The negative effects of cold exposure on the immune system have been demonstrated in blue tits [14], mice [7], and chickens [6, 11]. However, such negative effects have not been found on immunocompetence in studies in which bobwhite quail [13] and chickens [11, 81] were exposed to cold. Even positive effects of cold on immunity were reported in chickens [1, 81, 104] and prairie vole [118]. These contradictory results call for further studies on the effects of increased costs of thermoregulation on immune function. Moreover, earlier studies mostly lack information on different components of the immune system as most studies have monitored only a part of the immune system. Therefore, we hypothesized that cold exposure could differentially affect certain components of the immune system. Hence, both innate and adaptive immune components (Figure 1) were monitored weekly for 10 wk. In addition, we hypothesized that the cold exposure might have differential effects on animals with different genetic background. Hence, we studied the effect of cold exposure on two lines of chicken divergently selected for 23 generations for high (H line) and low (L line) antibody responses [57].

Life-history theory predicts that, when internal resources are limited, any significant increase of the investment in one vital process is likely to compromise other vital processes contributing to the animal's fitness [119]. The vertebrate immune system is one of the most intensively studied vital processes of life-history theory. It has been proposed to compete for nutrient demands with other vital processes such as thermoregulation, reproduction, and growth [20, 56]. The energetic trade-offs between vital processes can only be measured after elevating the energy requirement for different vital processes under the conditions that exhaust the ability to increase energy acquisition. Otherwise, the extra costs evoked may be fueled by increased rates of energy acquisition rather than its reallocation from other essential components of energy budget [26]. For this reason, we increased the energy expenditure of growing birds from two selected lines by cold exposure (energy for thermoregulation), and immunization (energy for immune response) while under restricted feeding conditions. This created a unique situation demanding a trade-off between thermoregulation, genetically driven

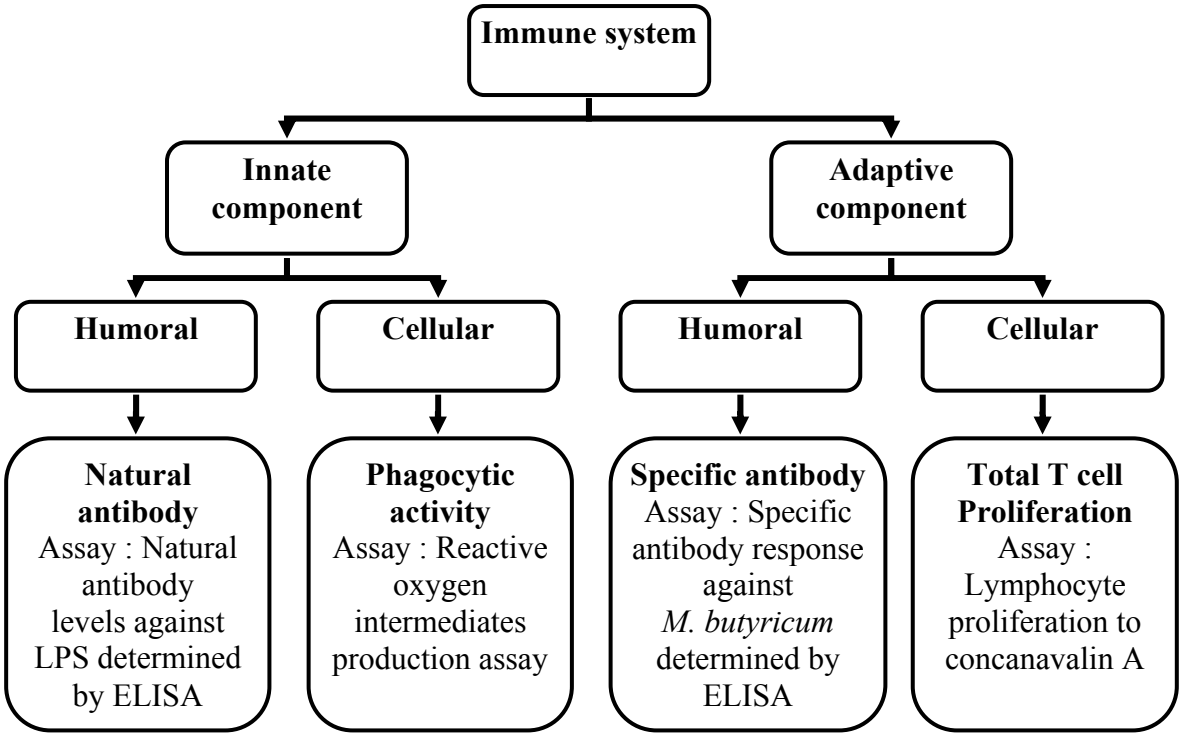

Figure 1. Schematic representation of arms of the immune system and assays used for measurement.

magnitude of immune response and growth. Similar attempts have been made by Cichon et al. [7] in mice. However, in that study authors evaluated only a specific humoral response to a novel antigen. Therefore, that study lacks information on whether a trade-off existed between the whole immune function and thermoregulation, or whether the trade-off existed only between the specific humoral response and thermoregulation. Apart from that, the cold stressed mice consumed almost double the amount of feed of the control mice. Therefore, feeding level might have interfered with the outcome as excess feed intake is known to influence immunocompetence [39].

The present experimental conditions mimic winter condition i.e. long period of low temperature with limited availability of food. The aspects addressed in the present study are of interest to both poultry farmers who might shift towards farming systems resembling more

natural conditions [104], and to ecologists wishing to understand the winter ecology. In addition to increased predation and starvation [120], reduced immunocompetence during cold winters may potentially affect disease risk and fitness, and hence avian population dynamics [13]. Furthermore, energetic stress is of general importance in ecological contexts [19, 22].

In the present study, apart from immune parameters, individual body weight was also monitored. That allowed us to quantify a trade-off between physiological traits such immunocompetence, growth and thermoregulation. To our knowledge the present study is the first of its kind which significantly challenged the energy budget of animals, and both innate and adaptive immune components were monitored. We also studied whether hosts mounting a high specific immune response were able to mount a strong nonspecific immune response. This would be expected if, for example, a strong nonspecific immune response reflected overall immunocompetence. Alternatively, specific defense against a pathogen could come at the price of reduced nonspecific immunity as expected if some kind of trade-off between these two arms of the immune system is involved.

6.3 Material and methods

Chickens

Forty 23-d-old Institute Selection Animal (ISA) Warren medium heavy layer pullets from two lines were used in this study. The two lines were divergently selected for 22 generations for high (H line) or low (L line) primary antibody responses at d 5 after intramuscular immunization with SRBC at 35 day of age [57]. Details regarding the immunological methods, the selection process and phenotypic differences of these lines have been described elsewhere [53, 81, 121].

The first 20 d after hatch chicks were fed *ad libitum* with a commercial diet (200g kg^{-1} crude protein, 2600 kcal kg^{-1} metabolizable energy) and were kept according to routine procedures. From 20 d of age, birds were fed once a day based on their body weight with the same commercial diet at 194.4 kcal $kg^{-0.75}d^{-1}$ (i,e 140% of $ME_{m,}$ minimum metabolic energy for maintenance) [55] for these selection lines. All birds had free access to water throughout the experiment. The L:D regime for both control and cold exposed birds was 14:10 (lights on at 05.00 h). The birds were vaccinated for Marek's disease, and infectious bronchitis at hatch and infectious bursal disease on day 15 of age. Experimental protocols were approved by the

Institutional Animal Care and Use Committee of Wageningen University (approval no. 2004022).

Experimental design

At 20 days of age, two groups of 10 hens each of H and L line were randomly assigned to control (C) or treatment group (CS). Both C and CS birds were housed in one of the two identical climate chambers in groups of 5 [58] which were maintained at 21.7±1.9^{o}C with relative humidity (RH) of 58±8% (control temperature). After a three days of acclimatization (23 d of age), the temperatures of one of the climate chambers was decreased rapidly to 10±0.4^{o}C with RH of 70±1%. After 6 wk of cold exposure, C and CS birds were immunized subcutaneously with 0.5 mL complete Freund's adjuvant (CFA, Difco, Detroit, MI) / bird. BW was recorded and one mL blood samples were collected weekly during 10 wk of cold exposure. Blood samples were used to measure lymphocyte proliferation to Concanavalin A (Con A, Sigma Chemical Co., St. Louis, MO) and reactive oxygen intermediates production during zymosan A (Sigma Chemical Co., St. Louis, MO) stimulation in whole blood assays. Natural antibody titers binding *Salmonella enteritidis* lipopolysaccharide (LPS, Sigma Chemical Co., St. Louis, MO) were measured in all samples. Plasma samples from d 0, 1, 7, 14 and 21 after immunization were used to measure specific antibody titers to *Mycobacterium butyricum* (Difco, Detroit, MI).

Assay for antibody level determination

Specific antibodies binding to *M. butyricum* or natural antibodies binding to LPS were determined in individual plasma samples by indirect two-step ELISA. Plates coated with 1 μg/mL *M. butyricum* or 4 μg/mL LPS, were incubated with serial twofold dilutions of plasma. Binding of chicken antibodies to the antigens was detected using 1:20,000 diluted conjugated secondary rabbit antibodies labeled with peroxidase (RACh/IgG_{H+L}/PO, directed to chicken total antibodies, Nordic, Tilburg, The Netherlands). After washing, tetramethylbenzidine and 0.05% H_2O_2 were added, and incubated for 10 min at room temperature. The reaction was stopped by adding 2.5 N H_2SO_4. Extinctions were measured with a Multiskan (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Titers were expressed as the log2 values of the

dilutions that gave extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of standard positive plasma present on every microtiter plate.

Assay for Lymphocyte proliferation to Con A

In vitro lymphocyte stimulation tests (LST) of whole blood were performed as follows. Aliquots of 100 μL of whole blood diluted with tissue culture medium (1:60) was added to 6 wells of 96-well microtiter plate. To the first 3 wells, 100 μL of 20 μg/mL Con A in tissue culture medium supplemented with 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 IU/mL penicillin was added. To the next 3 wells, 100 μL of tissue culture medium supplemented with 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 IU/mL penicillin was added and cultured during 48 hours at 41^{o}C in a humidified atmosphere with 5% CO_2. The last 12 hours before harvesting, cultures were pulsed with 0.5 μCi methyl-[^{3}H]-thymidine (ICN Biomedicals Inc. Aurora, OH). Thymidine uptake was determined with a Beckman beta-scintillation counter.

Results were expressed as mean stimulation indices (SI), calculated as SI = mean counts per minute (CPM) in mitogen stimulated cultures / mean CPM in unstimulated cultures.

Determination of reactive oxygen intermediates (ROI) production

The 'Nitroblue Tetrazolium (NBT, Sigma Chemical Co., St. Louis, MO) Assay' was used to measure ROI production by phagocytes in whole blood as described before [54] with slight modifications. One hundred microliters of 1:60 diluted heparinized blood and 50 μL of 2 mg/mL NBT in PBS were added to six wells of 96-well flat bottom microtiter plates. To the first three wells, 10 μL of 1 mg/mL of phagocyte stimulator, Zymosan A (prepared from *Saccharomyces cerevisiae*), in cell culture medium was added. To the next three wells, 10 μL of cell culture medium was added (non-Zymosan stimulated). Three additional wells were used as blanks and contained 100 μL of cell culture medium, 50 μL of 2 mg/mL NBT in PBS, and 10 μL of 1 mg/mL of Zymosan A, and three wells were used as reagent controls, containing 100 μL of 1:60 diluted heparinized blood, 10 μL of mg/mL of Zymosan A, and 50 μL of cell culture medium. After 1 hour of incubation at 41^{o}C and 5% CO_2 in a humidified atmosphere, the reaction was stopped with 100 μl 1N HCL. Then each plate was centrifuged and washed two times with PBS (10 min, 850 x g). Finally, 150 μl dimethylsulfoxide, were

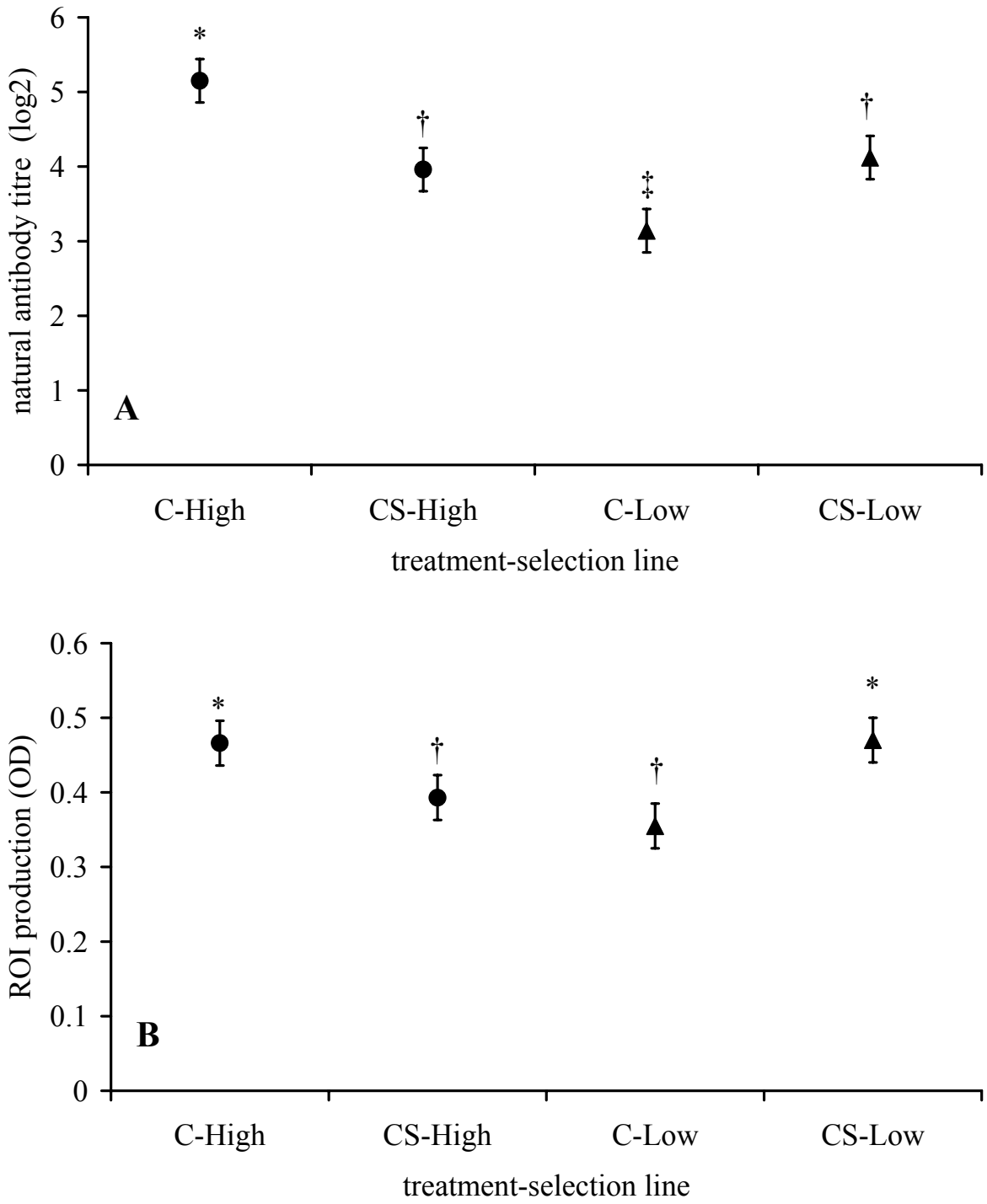

Figure 2. Effect of control (C) vs. cold stress (CS) on immunocompetence of chicken lines divergently selected on high and low antibody responses to sheep erythrocytes. (a) Natural antibody levels (±SE), (b) Reactive oxygen intermediates production (ROI) (±SE).

added, directly followed by 10 µl 1N KOH. Addition of dimethylsulfoxide caused a color change to blue, which was measured with a Multiskan at 690 nm. Mean absorbance of the non-zymosan stimulated samples was subtracted from the absorbance of the stimulated samples, as an indication of the reactive oxygen intermediates production during antigen digestion.

Statistical analysis

Differences in titers of plasma antibodies (specific and natural), lymphocyte proliferation to Con A, phagocytic activity and body weight gain were analyzed by a three-way ANOVA for the effect of treatment, line, time, and their interactions using the repeated measurement procedure using a 'bird nested within treatment and line' option. All analyses were according to SAS [59] procedures. Mean differences between treatments and lines were tested with Bonferroni's test.

6.4 Results

Natural antibodies binding Lipopolysaccharide (LPS)

CS had differential effects on natural antibodies in plasma binding LPS in both lines (interaction effect between treatment and line $F_{1,350} = 17.23$, $P < 0.001$). **N**atural antibodies in plasma binding LPS were significantly suppressed by CS in the H line birds, whereas natural antibody levels were significantly enhanced in the L line birds (Figure 2a). The level of natural antibodies binding LPS was significantly higher in H line than the L line birds (effect of line $F_{1,350} = 12.68$, $P = 0.001$, Figure 2a). There was a significant time ($F_{9,350} = 117.79$, $P < 0.001$) and its interaction effect with treatment ($F_{9,350} = 8.87$, $P < 0.001$), line ($F_{9,350} = 4.85$, $P < 0.001$) and both treatment and line ($F_{9,350} = 2.25$, $P = 0.02$).

Reactive oxygen intermediates production

CS had differential effect on ROI production in both lines (interaction effect between treatment and line $F_{1,347} = 12.25$, $p = 0.001$). CS had suppressive effect on ROI production in H line birds, whereas CS enhanced ROI production in L line birds (Figure 2b). The level of reactive oxygen intermediates production did not differ between H and L lines (line effect $F_{1,347} = 0.33$, $p = 0.567$). There was a significant time ($F_{9,347} = 63.48$, $P < 0.001$) and its

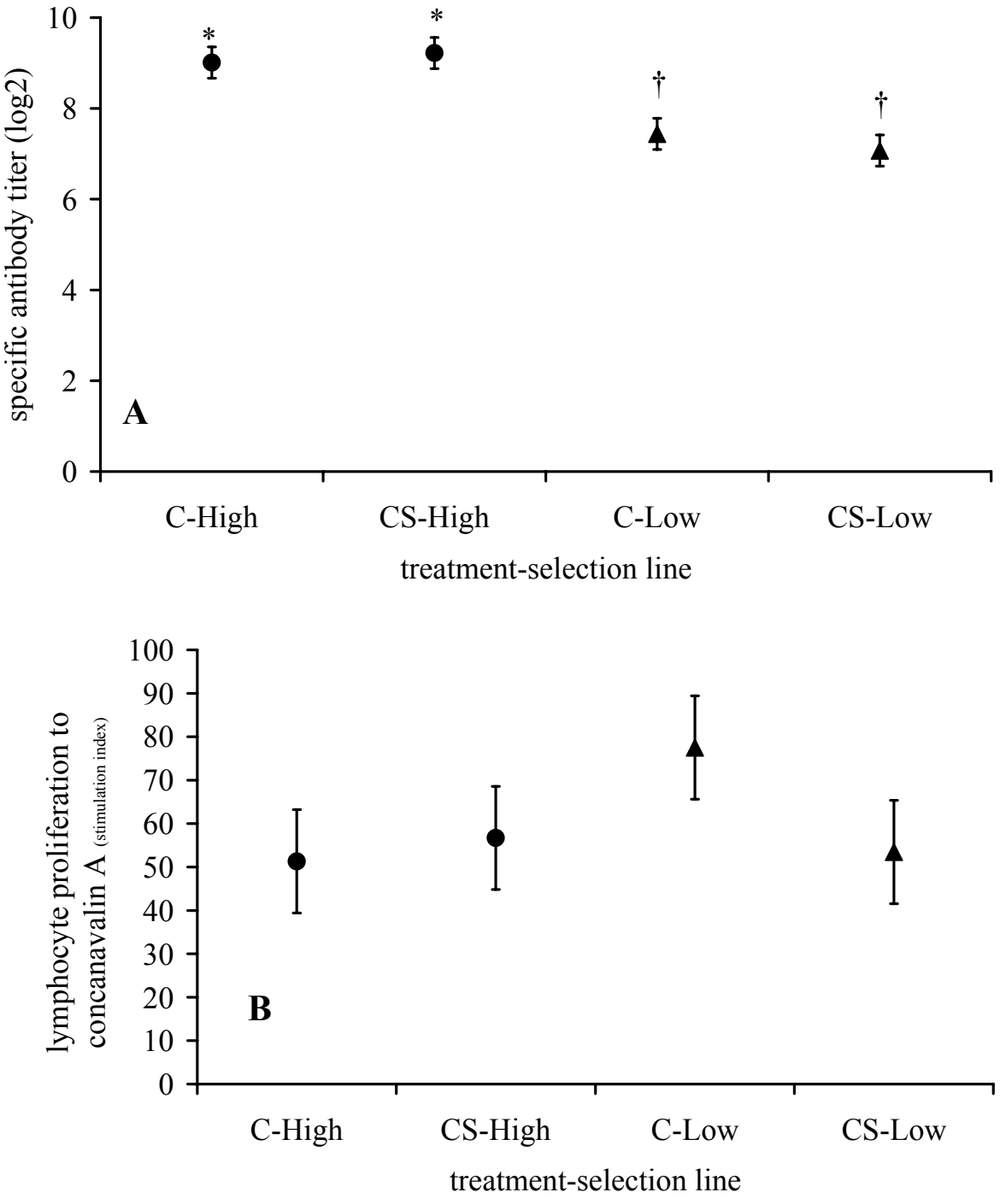

Figure 3. Effect of control (C) vs. cold stress (CS) on immunocompetence of chicken lines divergently selected on High and Low antibody responses to sheep erythrocytes. (a) Specific antibody responses (±SE) and (b) Lymphocyte proliferation to concanvalin A (±SE).

interaction effect with treatment ($F_{9,347}$ = 6.14, P < 0.001), line ($F_{9,347}$ = 6.81, P < 0.001) and combination of both treatment and line ($F_{9,347}$ = 1.91, P = 0.05).

Specific antibody responses to *Mycobacterium butyricum* (MB)

There was no significant effect of CS on specific antibody titers binding MB in birds of either line *(*$F_{1,175}$ = 0.08, P = 0.77, Figure 3a*)*. Levels of MB binding plasma antibody were significantly higher in H line than L line birds ($F_{1,175}$ = 36.32, P < 0.001, Figure 3a). There was a significant time effect ($F_{4,175}$ = 408.04, P < 0.001) but there was no significant interaction effects between CS and line ($F_{1,175}$ = 0.95, P = 0.34, Figure 3a), CS and time ($F_{4,175}$ = 1.76, P = 0.14), line and time ($F_{4,175}$ = 1.17, P = 0.33) or CS, line and time ($F_{4,175}$ = 0.21, P = 0.34).

Proliferation of whole Blood Lymphocytes in Response to Con A

Proliferation of whole blood lymphocyte in the presence of Con A was not significantly affected by CS ($F_{1,316}$ = 0.5, P = 0.485), line ($F_{1,316}$ = 0.75, P = 0.39) or CS and line interaction ($F_{1,316}$ = 1.23, P = 0.275). However, numerically enhanced proliferation of whole blood lymphocyte in the presence of Con A was seen in the H line subjected to CS, whereas numerically suppressed proliferation of whole blood lymphocyte in the presence of Con A was seen in the L line birds subjected to CS when compared with their respective control groups (Figure 3b). There was a significant time effect ($F_{8,316}$ = 20.78, P < 0.001) but there was no significant interaction effects between CS and time ($F_{8,316}$ = 1.24, P = 0.28), line and time ($F_{8,316}$ = 1.10, P = 0.36) or CS, line and time ($F_{8,316}$ = 0.95, P = 0.48)**.**

Body weight gain

CS resulted in significantly reduced body weight gain in both H and L line birds (treatment effect $F_{1,350}$ = 181.88, P < 0.001, Figure 4) but there was neither a significant line ($F_{1,350}$ = 0.77, P = 0.39) nor a significant interaction effect between treatment and line $F_{1,350}$ = 0.06, P = 0.81). There was a significant time ($F_{9,350}$ = 1577.99, P < 0.001) and its interaction effect with treatment ($F_{9,350}$ = 144.10, P < 0.001) but not with line ($F_{9,350}$ = 0.46, P = 0.90 or with the combination of both treatment and line ($F_{9,350}$ = 0.33, P = 0.96).

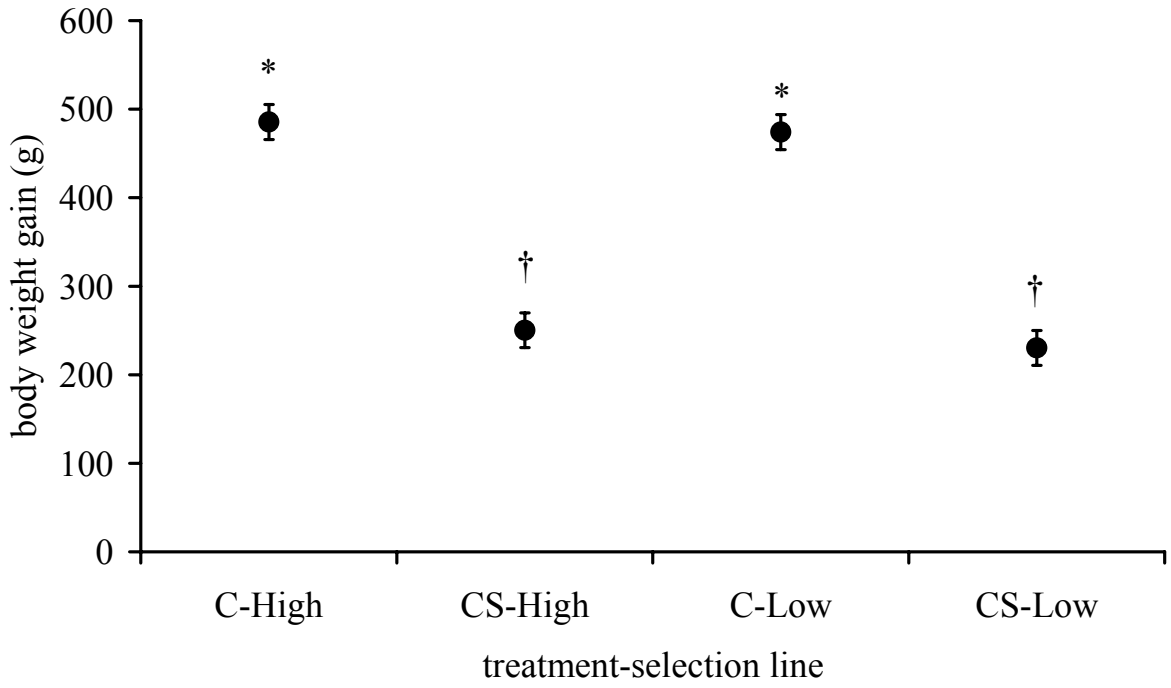

Figure 4. Effect of control (C) vs. cold stress (CS) on body weight gain (g) (±SE) of chicken lines divergently selected on high and low antibody responses to sheep erythrocytes.

6.5 Discussion

The present findings clearly demonstrate that prolonged exposure of chickens to cold induced differential effects on different arms of the immune system. Moreover, the effect of CS was strongly determined by the genetic background of the animal.

Natural antibodies (NAb) are 'non-specific' (background) antibodies present in plasma or sera of healthy non-immunized animals [61]. As part of the innate immunity, they facilitate antigen uptake, processing and presentation by B lymphocytes, induce or prevent auto-immune responses, clear lipopolysaccharides, lipoteichoic acid and debris, may protect against infection, or involved in a hypothetical immuno-regulatory idiotype-antiidiotype network [61, 112]. In birds, natural antibodies have been shown to be capable of modulating specific immune responses [122]. Next to the NAb, phagocytic activity which we measured as ROI production also forms another important component of the innate arm of the immune system. In contrast to specific humoral responses, in the present study CS suppressed innate immune responses (NAb and ROI) in H line, but were enhanced in L line. To our knowledge,

this is the first study in which effects of prolonged cold exposure on humoral component (NAb) of the innate immunity was monitored. It has been reported earlier that cold stress can increase phagocytic activity in mammals [123], which was the case in L line as well. Similarly, stress-induced increase in resistance of bird species to bacterial pathogens have been attributed to increased phagocytic activity [13, 124]. The genetically set differences in biological priority between the present lines may underlie the differential effect found in the present study. As L line birds have a significantly low ability to mount specific humoral immune response, innate immunity may become such an important determinant of fitness and such individuals can not afford to down-regulate this process, despite the conflicting demands of other life-history traits. This explanation suggests that innate immunity is a crucial life-history trait regardless of its cost in individuals with low ability to mount specific immune responses.

Immune responses to an antigen demonstrate clinically relevant alteration in an immunological response to challenge under well controlled conditions. Accordingly, they act as a proxy for responses to an infectious agent. Therefore, in individuals who produced delayed, weaker and shorter-lived immune responses to a novel antigen it is reasonable to assume these same individuals would also be slower to develop immune responses to other pathogens. Hence we measured specific antibody responses to an antigen in the present study. There was no effect of CS on specific antibody responses in either line. The present findings contradict with the previously reported increased energy expenditure that would have negative impacts on specific humoral immunocompetence [7, 14]. However, the present results are in agreement with previous findings [13, 81, 104, 125]. Our results are not in agreement with the suppressed disease resistance against parasites and extra cellular pathogens in avian winter ecology based on reduced humoral responses induced by low temperature alone.

Lymphocyte proliferation in response to mitogens is correlated with the ability of the host to mount a cellular immune response [126]. It has been suggested that differential reactivity to mitogens reflects either maturational or functional differences in the responsive lymphocytes. In the present study, the total cell mediated immune responses were suppressed irrespective of the genetic background of the animal in the early phase of CS treatment, whereas the effect diminished with time (Data not shown). Suppressive effects in the early phase of CS treatment on CMI has been reported earlier [6]. The physiologic mechanisms responsible for

the stress induced reduction in CMI responses are not well known. If serum corticosterone level was elevated at the early phase of the thermal stress, the following mechanisms could be postulated, a) corticosterone may have influenced the differentiation or inhibited the proliferation of cortisone-sensitive lymphocyte subpopulations involved in regulating these responses [127]. b) adrenal steroids could have caused an alteration in suppressor T cell or amplifier T cell activity [128]. Regardless of the underlying mechanisms, we found a temporarily suppressed cellular immunity, which may account for enhanced disease sensitivity during the early phase following a change in environmental conditions.

As expected, the present findings show that increased energy expenditure for thermoregulation under restricted feeding conditions resulted in significant decrease in body weight gain in both the lines.

We expected that H line birds subjected to CS should show highly suppressed antibody responses to a novel antigen when compared with low line birds, if mounting an antibody response entails metabolic costs and if there is a trade-off between immunocompetence and other life-history traits. However, there was no distinct suppression of specific antibody responses in H line birds subjected to CS. This finding suggests that the energetic costs involved in mounting a specific antibody response are not significant as proposed earlier [63, 64]. Furthermore, this trait may have higher genetically determined priority over growth and other aspects of somatic maintenance and repair including components of the immune system

Life-history trade-offs have been thought to result from competition among different traits for limited internal resources [129]. If internal resources are limited, an increase of resources allocated to one trait necessitates a decrease of resources available for other traits [26]. Thus reduced availability of energy/resources may substantially magnify a trade-off [130]. In the present experimental approach we limited the energy/nutrient availability by restricted feeding and also enhanced the energy allocated for thermoregulation by subjecting birds to cold stress and also enhanced the energy allocated for development of immune response by immunizing birds with nonpathogenic antigen. Thereby, we revealed rules governing the relative allocation of nutrients to different physiological processes [129]. As predicted, a life-history trade-off between growth and thermoregulation was seen. However, an expected trade-off between thermoregulation and immunocompetence was not seen, even though the energetic demand elicited by CS and the immunization with a non-pathogenic antigen used in

the present study were significant as compared with earlier studies [7, 14, 131]. Contradictory to the widely assumed negative effect of cold stress on immunity, we found even enhancement of innate immunity in L line birds after cold exposure. Thus, the present finding rules out the energetic explanation of possibility of trade-off between thermoregulation and immune response.

Cold stress did not affect specific immune responses and suppressed innate immune responses (NAb and ROI) in H line, whereas in L lines opposite effects were found. These findings provide an experimental support for trade-offs between specific and innate arms of immune system. Furthermore, this trade-off between different parts of the immune system is determined by the genetic background of an individual. The results are consistent with previously demonstrated concept of trade-offs between different arms of the immune system in bumblebees where strains with strong non-specific responses showed reduced specific responses [132].

In summary the present study clearly demonstrates that the cold exposure differentially affected arms of the immune system. Moreover, the effect was determined by the genetic background of the animal. The present findings suggest a trade-off between different arms of immune system. Therefore a thorough understanding of the whole immune status of an animal is required before reaching any conclusion relating to the trade-off between immunocompetence and other vital processes.

6.6 Acknowledgments

We thank Dr. Simon Verhulst and Prof. Paul Cotter for critical comments on the manuscript, members of ADP group for discussions and Mike G. Nieuwland and Ger De Vries Reilingh for technical assistance.

Chapter 7

Cold stress equally enhances in vivo pro-inflammatory cytokine gene expression in chicken lines divergently selected for antibody responses

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7.1 Abstract

The effects of cold stress, immunization and genetic selection on the expression of mRNA for cytokine genes in poultry have not been completely elucidated. Therefore, in the present experiment, using real-time quantitative RT-PCR, we evaluated the effect of cold stress and immunization with complete Freund's adjuvant (CFA) on expression of mRNA for pro-inflammatory (interleukin-1beta [IL-1β], IL-6, IL-12β), Th_1 (IFN-γ and IL-2), and Th_2 (IL-4 and IL-10) cytokine genes in peripheral blood leukocytes (PBL) of chicken lines divergently selected for either high or low antibody responses. Irrespective of the duration, cold stress enhanced expression of mRNA for IL-1β, IL-6, IL-12β and IL-4 cytokine genes in both selection lines. These results indicate that cold stress stimulates both the innate and parts of the adaptive cellular immune system. Immunization with CFA resulted in higher expression of mRNA for pro-inflammatory cytokines and lower expression of mRNA for both Th_1 and Th_2 cytokines.

7.2 Introduction

Cold stress (CS), a physical environmental stressor, has been shown to have variable modulatory effects on cells of the immune system in laboratory animals [133-137] and human[138, 139]. Similarly, effects of CS on immune cell functions, though inconsistent, were demonstrated in aves [6, 10-14]. Our recent attempts to understand the effects of CS on immune cell functions in poultry showed that CS does not affect specific antibody responses, but enhances phagocyte activity. CS also enhances or suppresses cell mediated immune responses depending on the duration exposure to CS and the time point at which this immune parameter is determined [81, 104]. Such effects of CS on cellular immunity were still found at considerable periods after termination of CS and returning to optimal temperatures [81, 104], suggesting irreversible priming of immune competence in these birds. CS suppressed plasma corticosterone levels depending on the duration of exposure to CS as well [140].

Cytokines play a key role in bidirectional communication between the neuro-endocrine and immune systems [141]. It has been suggested that the interplay between hormones and cytokines during thermal stress may influence immune homeostasis in response to environmental challenges [142]. In humans, CS was reported to enhance IL-6 [138, 139, 143], IL-2 [139] and TNF-α [143] cytokine levels. CS did not have a significant effect on IL-1β cytokine levels in man [139], while in mice, cold exposure enhanced IL-1 cytokine levels [136]. Most cytokines have pleiotropic or redundant functions, and the level of one cytokine is tightly regulated by other cytokines. For example, an increase in Th_2 cytokines (e.g. IL-4 and IL-10) can result in a decreased Th_1 cytokines (e.g. IFN-γ and IL-2) [144]. Thus, some factors elevated by stress exposure could be responsible, in part, for stress-induced suppression of other factors. Therefore, it is important to examine multiple cytokines in response to a given stressor to understand the role of cytokines in the physiological response to stress exposure. To date, most of the studies have focused on the effect of stress on only a few pro-inflammatory cytokines [145]. In poultry, the lack of molecular tools has limited the accumulation of information on effects of CS on cytokine profiles. The recent availability of molecular tools to measure cytokine gene expression in the chicken [146-148], facilitate studies on the effects of environmental stress on the host at a cellular and molecular level.

Genetic improvement has been used as one of the tools to enhance disease resistance in animal production [46]. In poultry, a popular approach has been to divergently select for

antibody production against different antigens [46]. Genetic selection for specific antibody responses has been reported to affect other immune responses such as cell mediated immune response [47-49] and natural antibody levels [50]. However, there is not much information available on the effect of genetic selection for antibody responses on expression of mRNA for cytokine genes.

The main aims of the present study were 1) to determine the effect of duration of CS on expression of mRNA for pro-inflammatory cytokine genes (IL-1β, IL-6, and IL-12β), Th_1 cytokine genes (IL-2, and IFN-γ), and Th_2 cytokine genes (IL-4 and IL-10) in chicken lines divergently selected for high and low antibody responses to SRBC, 2) to determine the effect of immunization with CFA, a Th_1 antigen, on expression of mRNA for number of these cytokine genes in the selected chicken lines, and 3) to determine the effect of genetic selection for specific antibody responses on expression of mRNA for a number of cytokine genes which are acting at the level of both innate as well as specific immunity in non-immunized birds.

7.3 Materials and methods

Chickens

Forty 23-d-old Institute Selection Animal (ISA) Warren medium heavy layer pullets from two lines were used in this study. The two lines were divergently selected for 22 generations for high (H line) or low (L line) primary antibody responses at d 5 after intramuscular immunization with SRBC at 35 day of age [57]. Details regarding the immunological methods, the selection process and phenotypic differences of these lines have been described earlier [53, 81, 121]. The H and L line birds selected for 22 generations had an average SRBC agglutination titer of 19.7 and 1.3, respectively.

The first 20 d after hatch chicks were fed *ad libitum* with a commercial diet (200g kg^{-1} crude protein, 2600 kcal kg^{-1} metabolizable energy), and were kept according to routine procedures for layers. From 20 d of age, birds were fed once a day with the same commercial diet at 80% *ad libitum* (160% of minimum metabolic energy for maintenance [149] for these selection lines). All birds had free access to water throughout the experiment. The L:D regime for both control and cold exposed birds was 14:10 (lights on at 05.00 h). The birds were

vaccinated against Marek's disease and infectious bronchitis at hatch, and infectious bursal disease on day 15 of age.

Experimental design

At 20 days of age, two groups of 10 hens each of the H and the L lines were randomly assigned to control (C) or CS (CS) group. C birds were kept according to standard procedures except for the feed. CS birds were housed in one of two climate chambers in subgroups of 5 which were maintained at 21.7 ± 1.9 ^{o}C with a relative humidity (RH) of 58 ± 8% (control temperature at which C birds were kept during the experimental period). After a three days of acclimatization (23 d of age), the temperature of the climate chambers was decreased rapidly to 10 ± 0.4 ^{o}C with a RH of 70 ± 1%. At 42 d of CS (65 d of age), both C and CS birds were immunized subcutaneously with 0.5 mL complete Freund's adjuvant (CFA), (Difco, Detroit, MI) / bird.

One mL blood samples were collected at d 7, 42 (prior to immunization) and 43 after CS from all individuals for quantification of cytokine mRNA expression levels. Leukocytes were separated from red blood cells in the blood samples using lymphocyte separation medium (Axis Shield, Oslo, Norway) by centrifugation for 90 sec at 11,000 rpm. The leukocytes, contained in the buffy coat, were washed 2 times in RPMI 1640 by centrifugation for 30 sec at 11,000 rpm, pelleted, and resuspended in an RNA stabilization reagent (QIAGEN Gmbh Hilden, Germany) and stored at -80 ^{o}C until the isolation of RNA.

Real-time quantitative RT-PCR

Total RNA was isolated from the peripheral blood leukocytes (PBL) using the commercially available RNeasy Mini Kit (QIAGEN Gmbh Hilden, Germany) according to the manufacturer's instructions. Isolated total RNA was eluted in RNase-free water and stored at -80 ^{o}C until use. Because of the practical constraints, only 7-8 samples out of 10 samples originating from each experimental group were used for quantification of cytokine mRNA levels.

Cytokine mRNA expression levels in chicken leukocytes were quantified using real-time quantitative RT-PCR. Real-time quantitative RT-PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen Inc, Valencia, CA). The RT-PCR mixture consisted of 12.5 µl

2x QuantitTect SYBR Green RT-PCR Master Mix, 0.25 µl QuantitTect RT Mix, 1 µL (0.2-0.6 µM) of each specific primers (Table 1), 1 µL of template RNA, and the final volume made up to 25 µL with RNase-free water. Reactions were run on the MJR Opticon 2^{TM} Continuous Fluorescence detector (Bio-Rad) with the following cycle profile: 1 cycle of 50 ^{o}C for 30 min, 95 ^{o}C for 15 min, and 40 cycles of 94 ^{o}C for 15 s, 59 ^{o}C for 30 s, and 72 ^{o}C for 30 s. At the end of the cycles, melting temperatures of the PCR products was determined between 70 ^{o}C and 90 ^{o}C. The Opticon Monitor Software 2.02 (Bio-Rad) was used for detection of fluorescent signals and melting temperature calculations.

Table 1. Real-time quantitative RT-PCR primers and their concentration used

RNA target		Primer sequence (5'-3')	Exon boundary	Accession No.[a]	Conc. used
28S	F	GGCGAAGCCAGAGGAAACT	-	X59733	0.6µM
	R	GACGACCGATTTGCACGTC			
IL-1β	F	GCTCTACATGTCGTGTGTGATGAG	5 / 6	AJ245728	0.4µM
	R	TGTCGATGTCCCGCATGA			
IL-6	F	GCTCGCCGGCTTCGA	3 / 4	AJ250838	0.2µM
	R	GGTAGGTCTGAAAGGCGAACAG			
IL-12β	F	TGTCTCACCTGCTATTTGCCTTAC	1 / 2	AJ564202	0.4µM
	R	CATACACATTCTCTCTAAGTTTCCACTGT			
IFN-γ	F	GTGAAGAAGGTGAAAGATATCATGGA	3 / 4	Y07922	0.6µM
	R	GCTTTGCGCTGGATTCTCA			
IL-2	F	TTGGAAAATATCAAGAACAAGATTCATC	2 / 3	AJ224516	0.6µM
	R	TCCCAGGTAACACTGCAGAGTTT			
IL-4	F	AACATGCGTCAGCTCCTGAAT	3 / 4	AJ621735	0.4µM
	R	TCTGCTAGGAACTTCTCCATTGAA			
IL-10	F	CATGCTGCTGGGCCTGAA	3 / 4	AJ621614	0.4µM
	R	CGTCTCCTTGATCTGCTTGATG			

[a]Refers to the genomic DNA sequence

Quantification was based on the increased fluorescence detected by the DNA Engine Opticon 2^{TM} (Bio-Rad) due to increased binding of intercalating SYBR Green I dye only to double stranded DNA during PCR amplification. Results are expressed in terms of the threshold cycle value (C_t), the cycle at which the change in the reporter dye (ΔR_n) passes the significance threshold. For the present work, the threshold values of ΔR_n for all reactions described are shown in Table 2.

Table 2. Standard curve data from real-time quantitative RT-PCRs on total RNA extracted from peripheral blood leucocytes

mRNA target	ΔR_n[a] significance threshold	Log dilutions	C_t values[b]	R^{2c}	Slope[d]
28S	0.015	10^{-1}-10^{-5}	3-9	0.98	-0.30
IL-1β	0.015	10^{-1}-10^{-5}	18-25	0.99	-0.29
IL-6	0.015	10^{-1}-10^{-5}	22-28	0.99	-0.25
IL-12β	0.015	10^{-1}-10^{-5}	20-27	0.95	-0.30
IFN-γ	0.015	10^{-1}-10^{-5}	23-29	0.98	-0.28
IL-2	0.015	10^{-1}-10^{-5}	19-28	0.97	-0.31
IL-4	0.015	10^{-5}-10^{-9}	19-27	1.00	-0.33
IL-10	0.015	10^{-1}-10^{-5}	20-27	1.00	-0.29

[a]ΔR_n = change in the reporter dye.
[b]C_t = threshold cycle value, the cycle at which the change in the reporter dye levels detected passes the ΔR_n.
[c]R^2 = coefficient of regression.

To generate gene-specific standard curves, plasmids containing each of the interleukin genes was *in vitro* transcribed with MEGAscript® (Ambion, Austion, TX) to get gene specific RNA and was serially diluted from 10^{-1} to 10^{-5}. To generate standard curve for 28S, pooled RNA sample was serially diluted and used. Each RT-PCR experiment contained triplicates of 30 test samples, one no-template-control, and a $\log_{10}$ dilution series. Regression analysis of the standard curve was used to calculate the slopes of the gene-specific $\log_{10}$ dilution series. To correct for differences between template RNA levels between samples within the experiment, first the Difference Factor for each sample was calculated by dividing the mean C_t value for 28S rRNA-specific product of a sample by the mean C_t value for 28S rRNA-

specific product of all samples. Second, the corrected cytokine mRNA per sample was calculated using the following formula:
[(40 – mean cytokine C_t sample) x cytokine slope] / [Difference Factor sample x 28S slope]

Statistical analysis

To test for the effects of treatment (C vs. CS, pre-immunization vs. post-immunization), line (high vs. low antibody responder to SRBC), time (7d vs. 42d of CS) and interaction between treatment, line and time, corrected cytokine mRNA values were analyzed by three-way ANOVA. All analyses were performed using the General Linear Model procedure of the JMP software [34]. The probability level (P) of <0.05 was considered significant.

7.4 Results

Effect of CS on cytokine mRNA levels

Irrespective of the duration of exposure, CS significantly enhanced IL-1β, IL-6, IL-12β and IL-4 cytokine mRNA levels in both lines (Fig. 1a & b, $P < 0.005$). However, the levels of cytokine mRNA levels measured in both control and treatment groups at 7 d after CS were significantly higher than the cytokine mRNA levels measured at 42 d after CS, with the exception of IFN-γ mRNA cytokine levels (Fig. 1a & b, $P < 0.05$). A three-way interaction between treatment, line and time was significant only for IFN-γ mRNA cytokine level ($F_{1, 60} = 4.3$, $P = 0.04$) (data not shown). CS significantly enhanced IFN-γ mRNA cytokine level only in H line birds subjected to CS for 42 d ($P < 0.05$).

Effect of immunization with CFA on cytokine mRNA levels

Immunization with CFA significantly enhanced IL-1β ($P < 0.001$) and IL-12β ($P < 0.05$) cytokine mRNA levels in both lines (Table 3). On the other hand immunization with CFA significantly suppressed IFN-γ, IL-2 and IL-10 cytokine mRNA levels in both lines (Table 3, $P < 0.001$).

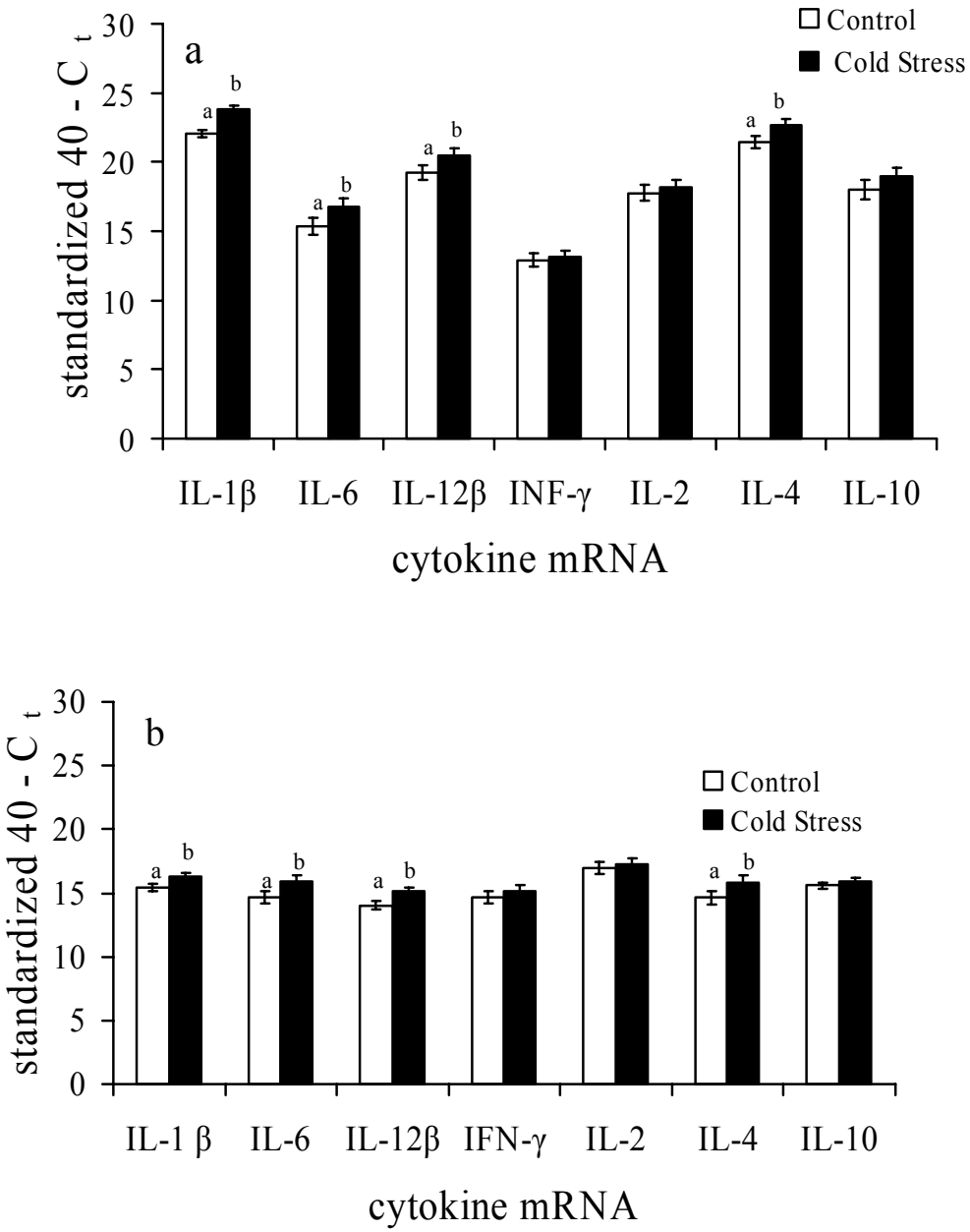

Figure 1. Quantification of cytokine mRNA levels in RNA extracted from semi purified peripheral blood leucocytes of chickens kept at optimal temperature (21.7 ± 1.9 ^{o}C) (white bars) or subjected to 7 (Fig. 1a) and 42 (Fig. 1b) d of cold stress (10 ± 0.4 ^{o}C) (black bars). The bars represent standardized values for cytokine mRNA levels subtracted from 40 (negative end point) and corrected for variation in input RNA measured by 28S rRNA levels. As values are subtracted from negative end point, higher values represent higher levels of cytokine mRNA levels. Error bars are S.E. for 7-8 samples from each treatment group. [a,b]Cytokine mRNA level differed significantly (P <0.05) between control and cold stress groups.

Effect of divergent selection for antibody responses on cytokine mRNA levels

There was a significant effect of genetic selection for high or low antibody responses on expression of mRNA for cytokine genes (Table 3, $P < 0.05$). The L line birds had significantly higher IL-1, IL-6, IL-12β, and IFN-γ cytokine mRNA levels when compared with the H line birds (Table 3, $P < 0.05$).

7.5 Discussion

The present experiment sought to examine whether durations of CS, immunization with CFA (Th_1 antigen), and genetic selection for specific antibody responses alter expression of mRNA for multiple pro-inflammatory, Th_1 and Th_2 type of cytokine genes in PBL. PBL are easily accessible immune cells when compared with splenocytes, as they can be harvested from experimental animals with a minimum damage to animals. Furthermore, studies with PBL facilitate gathering information on the immune status of the same animal over time. It has to be kept in mind, however, that the peripheral blood comprises multiple cell types and is usually not the site of an immune action, but may reflect the enhanced traffic of immune cells between the site of infection and lymphoid organs during an immune response. In the present study, the unstimulated cytokine response was measured to get a "snap-shot" of what cells are producing *in vivo* at the time of sampling. To our knowledge, the data provided herein are the first to examine whether CS affects peripheral expression of mRNA for cytokine genes in poultry. The principle findings of the present study are: a) irrespective of duration of CS and the genetic background of the birds, CS enhanced expression of mRNA for pro-inflammatory (IL-1β, IL-6, and IL-12β), and the Th_2 (IL-4) cytokine genes, b) immunization with the Th_1 inflammation inducing agent CFA, enhanced the expression of mRNA for IL-1β and IL-12β cytokine genes, but suppressed the expression of mRNA for IFN-γ, IL-2, and IL-10 cytokine genes, and c) the genetic selection for low specific antibody responses enhanced the expression of mRNA for pro-inflammatory and Th_1 related cytokine genes.

Pro-inflammatory cytokines are important in the recruitment of immune cells to the site of infection. The genes for the pro-inflammatory cytokines IL-1β, and IL-6 have been cloned and sequenced in chicken [150]. In chickens, pro-inflammatory cytokines may be released from variety of cells including circulating monocytes. In the present study, CS upregulated expression of both IL-1β, and IL-6 cytokine mRNA levels and this effect was not determined

by the duration of CS. The present findings are in agreement with the reported enhanced IL-1 levels in mice [136] and enhanced IL-6 levels in humans [138, 143] upon exposure to CS. However, one should keep in mind that upregulated mRNA expression for IL-1β, and IL-6 cytokine genes may not equate to enhanced levels of IL-1 and IL-6. In the present study immunization with CFA up regulated mRNA expression for IL-1β cytokine gene in PBL. Similar effect of immunization with CFA on IL-1β has been reported in the central nervous system of rodents [151]. In the rodent central nervous system, immunization with CFA also upregulated mRNA expression for IL-6 cytokine gene, however, we found no upregulation of mRNA expression for IL-6 cytokine gene in the present study using chicken PBL.

We also examined the effect of CS and immunization on mRNA expression for Th_1 and Th_2 immune response related cytokine genes in PBL. Th_1 and Th_2 refer to the T-helper cell type one and two respectively, differentiated from CD4+ cells when antigen-presenting cells such as macrophages present them with antigens. The Th_1 response is associated with the release of the cytokines IL-12β, IFN-γ, and IL-2, which seem to promote the growth of cytotoxic, killer, CD8+ T-cells and the inflammatory response of mononuclear leukocytes. These cells are the main weapons of the cellular immune response and are critical in locating and killing infected cells. The Th_2 response is associated with the release of IL-4, and IL-10. These cytokines tend to enhance the production of antibodies. In addition, Th_2 and Th_1 responses either mutually inhibit each other directly, or activate T-regulator cells to inhibit Th_1 and Th_2 inflammation. Our data show that CS upregulated mRNA expression for both IL-12β (Th_1 related) and IL-4 (Th_2 related) cytokine genes, suggesting that this physical stressor acts on APC in a non (-antigen) specific fashion. Immunization with the Th_1 antigen, CFA upregulated mRNA expression for IL-12β cytokine gene, but downregulated mRNA expression for the Th_1 cytokine genes, IFN-γ, and IL-2, and the Th_2 cytokine gene IL-10. Whether this observation reflects a type of immune modulation / regulation, or is related with re-localization of lymphocytes to the site of CFA-inflammation or lymphoid tissues remains to be established. However, the present data do not contradict our earlier findings indicating an enhancement of cellular (Th_1 related) immunity in birds after subjecting to CS in the same chicken lines [81].

The molecular signaling pathways involved in thermal stress and CFA-induced cytokine alterations in chicken are not completely known. The current findings suggest that the CS

Table 3. Quantification of cytokine mRNA levels[1] in RNA extracted from semi purified peripheral blood leucocytes of chicken lines selected for high (H) and low (L) antibody responses, collected at pre (Pre-immune) and 24 h post immunization (Post-immune) with complete Freund's adjuvant

Line	Treatment	Cytokine mRNA						
		IL-1β	IL-6	IL-12β	IFN-γ	IL-2	IL-4	IL-10
H	**Pre-immune**	15.05	14.44	13.77	14.07[b]	16.62	14.28	15.39
	Post-immune	16.66	13.85	14.39	10.65[c]	15.62	13.77	13.54
L	**Pre-immune**	15.76	14.96	14.28	15.29[a]	17.29	15.01	15.78
	Post-immune	17.09	14.80	15.24	10.81[c]	15.47	14.29	13.61
SEM		0.26	0.36	0.37	0.24	0.44	0.42	0.32
Effects								
Immunization		*** post>pre	NS (*P*=0.29)	* post>pre	*** pre>post	*** pre>post	NS (*P*=0.13)	*** pre>post
Line		* L>H	* L>H	* L>H	** L>H	NS (*P*=0.55)	NS (*P*=0.12)	NS (*P*=0.45)
Immunization x Line		NS (*P*=0.59)	NS (*P*=0.54)	NS (*P*=0.64)	* (*P*=0.03)	NS (*P*=0.35)	NS (*P*=0.79)	NS (*P*=0.61)

[a,-c]values with no common superscript differ significantly.

[1]values are standardized cytokine mRNA levels subtracted from 40 (negative end point) and corrected for variation in input RNA measured by 28S rRNA levels. As values are subtracted from negative end point, higher values represent higher levels of cytokine mRNA. Effect of immunization with complete Freund's adjuvant on cytokine mRNA levels was measured only in control birds.

SEM = Standard Error Mean, **P*<0.05, ***P*<0.005, ****P*<0.001, NS = Not significant, *N* = 30 (n = 7-8 birds).

enhances the cytokine mRNA levels of cytokines mainly produced by peripheral phagocytes or other antigen presenting cells. Recent studies with mice show that phagocytes are also a source of IL-4 apart from Th_2 cells [152]. Therefore, it is tempting to speculate that the enhanced IL-4 cytokine mRNA expression measured in the present study may be of innate origin rather than the adaptive immune system. The reasons why CS resulted in enhanced cellular (Th_1 related) but not humoral (Th_2 related) immunity in poultry [81, 104], though both IL-12β and IL-4 levels are enhanced after CS, are as yet unknown.

As CFA is well known to induce Th_1 type of immune response, it was expected to find enhanced mRNA expression for cytokines produced by monocytes (IL-1β, and IL-12β) and Th_1 cells (IFN-γ, and IL-2) upon immunization. The effects of CFA immunization on expression of mRNA for IL-1β, and IL-12β cytokine genes were thus in agreement with the expected results.

The effects of CS and immunization with CFA were determined in chicken lines selected for 22 generations for high or low antibody responses against SRBC. Divergent selection for antibody responses did not affect cytokine mRNA levels when subjected to CS. However, selection for antibody responses significantly affected cytokine mRNA levels of pro-inflammatory and Th_1 cytokines upon immunization with CFA. When compared with the H line, the L line had higher cytokine mRNA levels of IL-1β, IL-6, IFN-γ and IL-12β. This suggests that the selection for low antibody responses could have enhanced the IL-12β cytokine production capacity of monocytes and the IFN-γ cytokine production capacity of Th_1 cells in the L line. The higher production of IL-12β in the L line may direct more T-helper cells to become Th_1 rather than Th_2 thus contributing more to cellular immunity as frequently found in the L line. Further studies are, however, needed to characterize the cytokine production capacity of innate and adaptive immune cells from these selection lines upon stimulation with Th_1 and Th_2 type of antigens.

In conclusion, CS upregulated the mRNA levels of pro-inflammatory cytokines in both lines. The low level of mRNA expression of Th_1 and Th_2 cytokine mRNA after immunization with CFA could be due to re-localization of circulating T and B cells into lymphoid organs such as the spleen, and may therefore not reflect the functional immune status of the birds. Future studies should consider evaluating the effect of immunization on expression of mRNA for cytokine genes in PBL as well as cells from other lymphoid tissues. Such studies might

reveal the reason why CS although enhancing both IL-12β and IL-4 results in enhanced cellular immunity but not humoral immunity in poultry [81, 104]. Furthermore, the differences in cytokine production capacity of monocytes and Th_1 cells in these selection lines may underlie the phenotypic differences found in adaptive immune responses.

7.6 Acknowledgements

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Chapter 8

General Discussion

8.1 Abstract

The main aim of this thesis was to understand the immunological adaptive capacity of chicken lines from different genetic background to stress. To achieve the above aim, chicken lines divergently selected for production of either high or low antibody responses were exposed to cold stress (a model environmental stress) and their ability to mount specific antibody responses was evaluated. The aim was achieved by addressing the following hypotheses 1. “Effects of stress on immunity depend on type of stress, duration of exposure to stress, relative time point of measurement of immunity to the termination of stress exposure, the immune component measured and the genetic background of the animal”. 2. “Genetic selection for a trait (e.g. antibody responses) may program an individual to allocate a large portion of its resources to that demand, leaving it lacking the ability to respond to other demands”. 3. “Birds adapt to cold stress by suppressing energy spent for growth and immunocompetence and reallocating the released energy for increased requirement for thermoregulation”. 4. “Trade-offs exist between growth, immunocompetence and thermoregulation”. In this chapter, the results obtained from series of studies described in previous chapters are discussed in the context of above proposed aim and hypotheses. At the end of this chapter conclusions are drawn based on the results reported in this thesis and suggestions are listed for future studies.

8.2 Stress and immunity

The term stress has been used so broadly in biology that, no clear definition of stress has emerged. Unlike most diseases, stress has no defined aetiology or prognosis. For this reason, our intuitive feelings about stress often guide our use of the term [153]. In this thesis, stress is defined as adverse effects of the environment or management system which force changes in an animal's physiology or behavior to avoid physiological malfunctioning and assists the animal in coping with its environment. It is generally assumed that stress impairs the immune system, but the truth is probably much more complicated. Certainly, chronic stress is unhealthy, but the mechanisms involve much more than suppression of immune function [117, 154]. The dogma that stress suppresses immunity is to some extent based on the well-established immunosuppressive effects of glucocorticoids [155]. However, pharmacological doses used in most of the studies do not allow simple extrapolation to the normal physiological state. So far, vast numbers of studies have been conducted to unravel the effects of stress on the immune system in both domesticated and wild birds. However, the outcomes of those studies are not consistent. As mentioned in the introduction of this thesis, lack of consistent responses have been attributed to 1. type of stress, 2. duration of stress, 3. relative time point of measurement of immune parameters with stress, 4. component of the immune system measured, and 5. the genetic background of experimental animals used. To understand the effects of above variations, series of experiments were conducted using cold exposure as an environmental stress model. Cold stress was chosen as a model stress in this thesis because, the physiological responses it induces to attain homeostasis are of high priority and energy demanding in homeotherms [7].

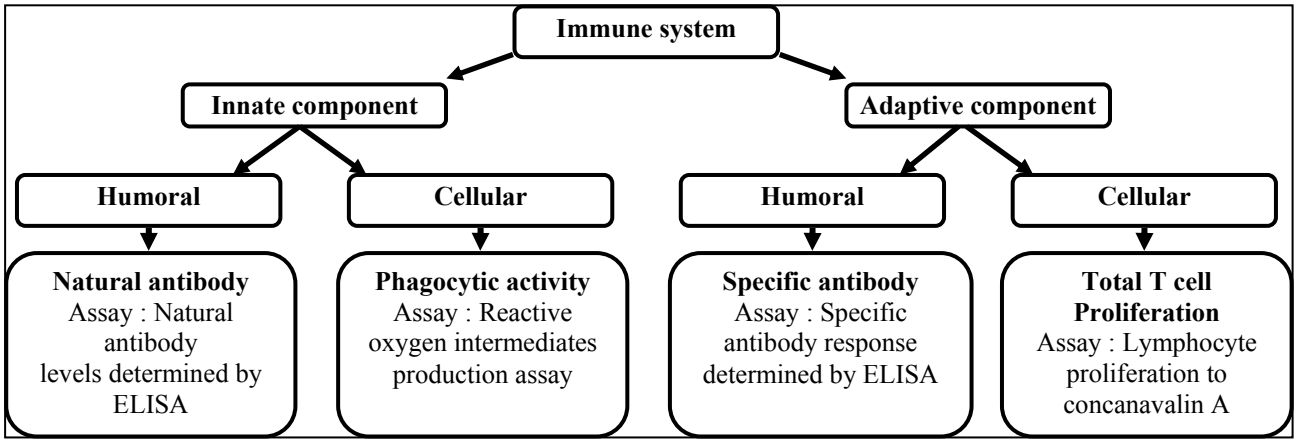

Figure 1. Schematic representation of the immune system components and assays used for measurement.

First, effects of CS and FR on the different immune system components (Fig. 1) and plasma corticosterone levels of chickens originating from two genetic backgrounds were evaluated (chapters 2 to 6). In general, both CS and FR enhanced innate immunity (ROI) but did not affect specific humoral immunity (Table 1). Similar effects of various kinds of stress on innate immune system (ROI) has been reported in other vertebrates [117, 156]. The determined effects of CS on cytokine gene expression levels (chapter 7) also supported the above findings. The complete molecular mechanisms underlying stress induced enhancement of innate immunity were not studied in this thesis. However, it is tempting to speculate that stress, irrespective of its type, may induce similar physiological responses (e.g. stress induced serum proteins such as heat shock proteins) to prepare the host innate immune system for impeding danger or non-optimal conditions as demonstrated in other vertebrates [117, 156]. A stress-induced enhancement of innate immune function makes sense when viewed from an evolutionary perspective. An acute stress response is an evolutionarily adaptive psychophysiological survival mechanism [157]. Since stressful natural encounters often result in wounding and infection, it is unlikely that eons of evolution would select for a system exquisitely designed to escape the jaws and claws of a lion to only have it eaten inside out by pathogens [117]. In general, CS and FR had differential effect on cell-mediated immunity and corticosterone levels. Cold stress enhanced cell-mediated immunity but suppressed plasma corticosterone levels, whereas FR had opposite effects. Metabolic stress and psychological stress have differential effects on plasma corticosterone levels [32, 116]. It also been reported that the severe FR induces more psychological stress rather than metabolic stress [115]. Therefore, CS and FR might have induced different nature of stress resulting in suppressed (CS) or enhanced (FR) corticosterone levels. The correlated changes in plasma corticosterone levels and cell-mediated immunity reported in chapters 3 & 5 suggests that the effects of both CS and FR found on cell-mediated immunity are determined by the plasma corticosterone levels.

Second, effects of different durations of CS on chickens originating from two genetic backgrounds were evaluated (chapters 2, 4 & 6) and the findings are summarized in table 1. Durations of CS had differential effects on various immune parameters measured. Innate immunity was enhanced in both lines irrespective of duration of CS. Only a long duration of CS (1 wk) exposure enhanced cell-mediated immunity (Table 8.1), and reduced body weight

gain in both lines. However, specific antibody responses were not affected by CS. Enhanced cell-mediated immune responses were correlated with suppressed corticosterone levels (Table 8.1). Higher levels of triiodothyronine were seen only in birds exposed to CS for a short duration, but no correlation with any immune parameters was observed (chapter 3). The results obtained from these studies clearly demonstrate that effect of stress depends on the immune component measured and the duration of exposure

Table 1. Summarized effects* of cold stress (10°C, CS) and feed restriction (60% *ad libitum*, FR) on immune parameters such as a) innate humoral immunity (Nab, natural antibody levels), b) innate cellular immunity (ROI, reactive oxygen intermediates), c) adaptive humoral immunity (SAb, specific antibody levels) and d) cell-mediate immunity (CMI) and endocrine parameter such as plasma corticosterone (CORT) level.

Duration	Immune parameter								Endocrine parameter	
	NAb		ROI		SAb		CMI		CORT	
	CS	FR	CS	FR	CS	FR	CS	FR	CS	FR
Short (<1 wk)	↑	-	↓ / ↑	-	NS	-	↓ / ↑	-	↓	-
Long (1 wk)	NS	-	NS	-	NS	-	↑	-	↓	-
Prolong (> 1 wk)	↓H / ↑L	↓	↓H / ↑L	↑	NS	NS	↑H / ↓L*	↓	-	↑

*All the studies were conducted using chickens lines which were divergently selected for either high (H) or low (L) antibody responses. Detailed experimental design, methods, and the effects are described in chapters 2-6. ↑ = enhancement significantly, ↓ = suppression, NS = no significant effect, - = not determined, * = effect of CS on both lines was not significant statistically. Effect considered significant when $P<0.05$.

Third, effects of CS on different immune parameters, measured at various time points after termination of CS were evaluated (chapter 4). The effects of CS did not differ from the findings reported above. In this study enhancing effects of CS (1 wk) on cell-mediated immunity were seen only after 21 days post CS suggesting the importance of relative time point of measurement of this immune parameter to the treatment. However, such effects were not seen on any other immune parameters such as natural and specific antibody levels.

As summarized in previous paragraphs both CS and FR had differential effect on different immune components but effects of CS and FR did not differ between chicken lines (chapters 2, 4 & 6). However, effects of prolong CS described in chapter 7, on innate (Nab and ROI) and cell-mediated immunity (CMI) differed between H and L (Table 1). The above finding

suggests that the genetic background of an animal may not determine the effects of short term CS and FR on immunocompetence.

8.3 Effects of genetic selection for a trait on physiological adaptive capacity

In the present thesis, H and L lines were used to address the hypothesis proposed by Beilharz [17] that "selection for a trait may program an individual to allocate a large portion of its resources to a demand, leaving it lacking in ability to respond to other demands". These birds were genetically selected to produce either high or low antibody responses against non-pathogenic antigen, sheep red blood cells [57]. These birds also differ significantly in mounting specific antibody responses against many other antigens [48]. If the above hypothesis is true, then upon exposure of both lines to CS or severe FR, H lines birds should have fewer resources available for thermoregulation and growth than L line birds, and hence should show low growth and / or survival rates under CS conditions.

Surprisingly, we did not find significant differences either in growth or survival rates between H and L lines upon exposure to CS or severe FR (chapters 2-6). Irrespective of exposure to CS or FR, H lines produced higher specific antibody responses against keyhole limpet haemocyanin (Th-2 type of antigen, chapter 2 & 4) and *Mycobacterium butyricum* (Th-1 type of antigen, chapter 5). It is important to note that both CS and FR substantially reduced body weight gain in both lines (chapters 5 & 6). As birds were restricted fed, upon cold exposure, they were not able to cover the increased costs of thermoregulation by increasing food consumption. The above findings suggest that mounting specific antibody responses has high priority over other traits, irrespective of its energetic costs. It is difficult to estimate direct energetic costs of mounting an immune response. However, indirect estimates of energetic costs for mounting specific antibody responses have reported low energetic costs [14, 64]. A similar attempt has been made by Ksiazek et al., [131] using two lines of laboratory mice selected for high basal metabolic rate (H-BMR) and low basal metabolic rate (L-BMR). At room temperature, H-BMR mice produced significantly lower antibody responses against SRBC than L-BMR mice. They had expected that the antibody responses to SRBC of cold exposed H-BMR line should be suppressed, because those mice are already burdened with the high energetic costs of maintenance of their bigger internal organs. Alternatively, they had predicted that the cold-elicited energetic demands related to up-regulation of metabolic machinery to be especially significant in L-BMR line. If so, the high

costs of rebuilding their machinery to meet high energetic demands of thermoregulation may cause reallocation of resources from the immune function, resulting suppressed antibody responses. However, such suppression of specific antibody responses against sheep red blood cells was not found in both lines. The above findings suggest that a genetic selection for a trait (e.g. specific antibody responses) may not affect short-term immunological adaptive capacity of animals.

8.4 Adaptation to CS by reallocation of resources between life traits

Animals often have to survive dynamic environmental conditions both in nature as well as in farms. Either a behavioral or a physiological change that allows animals to cope with a changed condition is called adaptation. The physiological changes to external stresses can result in either short term or long term adaptation. Among those, short-term physiological adaptive responses have greater importance for animal husbandry practices, because short-term adaptation affects individual organisms during their lifetimes, while long-term adaptation is an intergenerational phenomenon which operates on species, not on individuals. Therefore, in the present thesis an attempt has been made to understand the short-term physiological adaptive responses in chickens upon exposure to CS.

Short-term physiological adaptive responses of birds to CS are complex and may typically involve adjustments in thyroid metabolism [158], such as elevation of serum T_3 levels and decline in serum T_4 levels [159]. Although T_3 increased in chickens exposed to very short duration of CS (chapter 3), T_4 levels remained unchanged. In addition to thyroid metabolism, increased adrenal activity resulting in higher plasma corticosterone levels was reported in chickens subjected to CS [11]. Contradictory to the previous report, we found suppressed serum corticosterone upon exposure to CS (chapter 3). Both thyroid as well as adrenal hormones can influence both immune system and also the metabolic status of an animal [9, 74], but we did not find such correlations (chapter 3).

Stress-induced increased resistance of birds to challenge with bacterial pathogens is a commonly observed phenomenon. For example, cold stress, food restriction, corticosteroid injection, social stress, and handling stress can increase resistance of poultry to *Staphylococcus aureus*, *Escherichia coli*, and *Pasteurella multocida* [160-162]. In those studies, stress-induced enhanced resistance of birds to bacterial pathogens has been attributed

to increased phagocytic activity [124]. Similarly, we found enhanced phagocytic activity upon exposure to CS (chapters 2, 4, 5 and 6). Cold stress did not affect specific antibody responses, and even enhanced cell-mediated immunity. These findings are in agreement with previous reports [1, 13]. As expected CS reduced body weight gain of birds (chapters 2, 4 & 6).

One of the hypothesis addressed in this thesis was, that birds adapt to CS by reallocation of resources from body weight and the immune system to thermoregulation. If this hypothesis is true then, CS should have suppressed both immune competence and body weight gain in these chicken lines. However, expected suppressive effects of CS were only seen on body weight gain. Contradictory to the expected suppression of various immune parameters in birds exposed to CS, both innate and CMI were enhanced. These findings suggest that birds adapt to CS by reallocation of resources from body weight gain to thermoregulation and immune parameters.

8.5 Resource based trade-offs between immunity, thermoregulation and growth

Life-history trade-offs have been thought to result from competition among different traits for limited internal resources [129]. If internal resources are limited, an increase of resources allocated to one trait necessitates a decrease of resources available for other traits [26]. Thus, reduced availability of energy/resources may substantially magnify a trade-off [130]. Using the experimental approaches described in chapters 2, 4 & 6, we limited the energy availability for birds by restricted feeding and enhanced the energy requirement for thermoregulation and immune response. Thereby, we attempted to reveal the rules governing the relative allocation of nutrients to different physiological processes such as thermoregulation, growth and immune responses [129]. As predicted, a life-history trade-off between growth and thermoregulation was seen (reduction in growth upon exposure to CS, chapters 2-6). However, an expected trade-off between thermoregulation and immunocompetence was not seen, even though the energetic demand elicited by CS and the immunization with a non-pathogenic antigen used in the present study were significant as compared with earlier studies [7, 14, 131]. Contradictory to the widely assumed negative effect of CS on immunity, we found even enhancement of innate immunity (chapters 2, 4 & 5) and cell-mediated immunity in birds subjected to CS (chapters 2-4). Thus, the present finding rules out the energetic explanation of possibility of trade-off between thermoregulation and immune response.

As described in chapter 6, exposure to cold for a long duration enhanced cell-mediated immunity and suppressed innate immune responses (NAb and ROI) in H line, whereas opposite effects were found in L lines (Table 1). These findings provide an experimental support for trade-offs between adaptive and innate arms of the immune system. Furthermore, this trade-off between different parts of the immune system is determined by the genetic background of an individual. The results are consistent with the previously demonstrated concept of trade-offs between different arms of the immune system in non-vertebrate such as bumblebees, where strains with strong non-specific responses showed reduced specific responses [132].

8.6 General conclusions

The following conclusions could be drawn based on the results obtained from the studies described in the present thesis,

- CS and FR enhanced innate immunity, irrespective of duration of exposure.
- Long duration of exposure to CS enhanced adaptive cell-mediated immunity, but severe FR suppressed adaptive cell-mediated immunity.
- Chickens adapt to CS by reallocation of resources from body weight gain to thermoregulation and immune parameters.
- Genetic selection for a health trait such as immunocompetence may not affect moderate stress tolerance and short-term physiological adaptive capacity of chickens.
- Trade-off between thermoregulation and immune response were not found. However, results suggest a trade-off between the arms of the immune system.

8.7 Future studies

- Future studies should consider elucidating the immunological mechanisms through which stress enhances innate immunity. Findings of such studies may pave the way for developing biomedical treatments designed to harness endogenous physiological mediators to selectively augment immune responses during vaccination or infections in chickens.
- In the present thesis a constant temperature stress model was used to study the immunological adaptive capacity of chicken lines. It is interesting to study how fluctuating environmental temperatures affect immunological adaptive capacity of these chicken lines.

- Natural predictable cues (e.g. short day length during winter) for adverse environmental conditions are also reported to have impact on physiology of animals. Therefore, it might be interesting to study combined effects of natural cues with the adverse environmental conditions on the immunological adaptive capacity of these chicken lines.

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Summary

Summary

The main aim of this thesis was to understand the immunological adaptive capacity of chicken lines from different genetic background to stress. To achieve this aim, chicken lines divergently selected for production of either high or low antibody responses were exposed to model environmental stress and their ability to mount specific antibody responses was evaluated. In this thesis cold stress (10^{o}C) is used as a model environmental stressor and feed restriction as a model social stressor. The aim was achieved by addressing the following hypotheses 1. "Effects of stress on immunity depend on type of stress, duration of exposure to stress, relative time point of measurement of immunity to the termination of stress exposure, the immune component measured and the genetic background of the animal". 2. "Genetic selection for a trait (e.g. antibody responses) may program an individual to allocate a large portion of its resources to that demand, leaving it lacking the ability to respond to other demands". 3. "Birds adapt to cold stress by suppressing energy spent for growth and immunocompetence and reallocating the released energy for increased requirement for thermoregulation". 4. "Trade-offs exist between growth, immunocompetence and thermoregulation".

First, effects of CS and FR on the different immune system components and plasma corticosterone levels of chickens originating from two genetic backgrounds were evaluated and described in chapters 2 to 6. In general, both CS and FR enhanced innate immunity (ROI) but did not affect specific humoral immunity (Table 1). The determined effects of CS on cytokine gene expression levels (chapter 7) also supported the above findings. In general, CS and FR had differential effect on cell-mediated immunity and corticosterone levels. Cold stress enhanced cell-mediated immunity but suppressed plasma corticosterone levels, whereas FR had opposite effects. The correlated changes in plasma corticosterone levels and cell-mediated immunity reported in chapters 3 & 5 suggests that the effects of both CS and FR found on cell-mediated immunity are determined by the plasma corticosterone levels.

Second, effects of different durations of CS on chickens originating from two genetic backgrounds were evaluated and described in chapters 2, 4 & 6 and the findings are summarized in table 1. Durations of CS had differential effects on various immune parameters measured. Innate immunity was enhanced in both lines irrespective of duration of CS. Only a long duration of CS (1 wk) exposure enhanced cell-mediated immunity (Table 1), and

reduced body weight gain in both lines. However, specific antibody responses were not affected by CS. Enhanced cell-mediated immune responses were correlated with suppressed corticosterone levels (Table 1). Higher levels of triiodothyronine were seen only in birds exposed to CS for a short duration, but no correlation with any immune parameters was observed (chapter 3). The results obtained from these studies clearly demonstrate that effect of stress depends on the immune component measured and the duration of exposure.

Duration	Immune parameter								Endocrine parameter	
	NAb		ROI		SAb		CMI		CORT	
	CS	FR	CS	FR	CS	FR	CS	FR	CS	FR
Short (<1 wk)	↑	-	↓ / ↑	-	NS	-	↓ / ↑	-	↓	-
Long (1 wk)	NS	-	NS	-	NS	-	↑	-	↓	-
Prolong (> 1 wk)	↓H / ↑L	↓	↓H / ↑L	↑	NS	NS	↑H / ↓L*	↓	-	↑

Table 1. Summarized effects of cold stress (10^{o}C, CS) and feed restriction (60% *ad libitum*, FR) on immune parameters such as a) innate humoral immunity (Nab, natural antibody levels), b) innate cellular immunity (ROI, reactive oxygen intermediates), c) adaptive humoral immunity (SAb, specific antibody levels) and d) cell-mediate immunity (CMI) and endocrine parameter such as plasma corticosterone (CORT) level. All the studies were conducted using chickens lines which were divergently selected for either high (H) or low (L) antibody responses. Detailed experimental design, methods, and the effects are described in chapters 2-6. ↑ = enhancement significantly, ↓ = suppression, NS = no significant effect, - = not determined, * = effect of CS on both lines was not significant statistically. Effect considered significant when $P<0.05$.

Third, effects of CS on different immune parameters, measured at various time points after termination of CS were evaluated and described in chapter 4. The effects of CS did not differ from the findings reported above. In this study enhancing effects of CS (1 wk) on cell-mediated immunity were seen only after 21 days post CS suggesting the importance of relative time point of measurement of this immune parameter to the treatment. However, such effects were not seen on any other immune parameters such as natural and specific antibody levels.

As summarized in previous paragraphs both CS and FR had differential effect on different immune components but effects of CS and FR did not differ between chicken lines (chapters 2, 4 & 6). However, effects of prolong CS described in chapter 7, on innate (Nab and ROI) and cell-mediated immunity (CMI) differed between H and L (Table 1). The above findings

suggest that 1. The genetic background of an animal may not determine the effects of short term CS and FR on immunocompetence. 2. Artificial genetic selection for a trait (e.g. antibody responses) may not affect short term physiological adaptive capacity of chickens.

One of the hypothesis addressed in this thesis (chapter 2, 4 & 6) was, that birds adapt to CS by reallocation of resources from body weight and the immune system to thermoregulation. If this hypothesis is true then, CS should have suppressed both immune competence and body weight gain in these chicken lines. However, expected suppressive effects of CS were only seen on body weight gain. Contradictory to the expected suppression of various immune parameters in birds exposed to CS, both innate and CMI were enhanced. These findings suggest that birds adapt to CS by reallocation of resources from body weight gain to thermoregulation and immune parameters.

As described in chapter 6, exposure to cold for a long duration enhanced cell-mediated immunity and suppressed innate immune responses (NAb and ROI) in H line, whereas opposite effects were found in L lines (Table 1). These findings provide an experimental support for trade-offs between adaptive and innate arms of the immune system. Furthermore, this trade-off between different parts of the immune system is determined by the genetic background of an individual.

Important conclusions drawn based on the results described in thesis are,

- CS and FR do not affect specific humoral immunity but enhance innate immunity.
- Genetic selection for a health trait such as immunocompetence may not affect moderate stress tolerance and short-term physiological adaptive capacity of chickens.
- Trade-off between thermoregulation and immune response were not found. However, results suggest a trade-off between the arms of the immune system.

Samenvatting (summary in Dutch, translated by Dr. Aart Lammers)

Het doel van het in dit proefschrift beschreven onderzoek was het verkrijgen van meer inzicht in de adaptieve capaciteit van het immuunsysteem van pluimvee onder invloed van omgevingsstressoren. Voor dit onderzoek werden kippenlijnen gebruikt die divergent waren geselecteerd op het vermogen om hoge- of lage antilichaam niveaus te genereren na immunisatie met een antigeen. Deze 2 kippenlijnen werden blootgesteld aan koude stress (10 °C) als een model voor omgevingsstress en werden beperkt gevoerd als een model voor sociale stress. In het onderzoek zijn de volgende hypotheses getest: 1. "De effecten van stress op de immuniteit zijn afhankelijk van het type stress, de tijd van blootstelling aan de stressor, het relatieve tijdstip van de meting van de immuniteit ten opzichte van de beëindiging van de stress, de immuunfunctie die wordt gemeten en de genetische achtergrond van het dier". 2. "Genetische selectie voor een eigenschap (b.v. antilichaam productie) bepaald welke prioriteiten een individu stelt met betrekking tot de verdeling van zijn "resources" in respons op een toegenomen behoefte voor die bepaalde functie, waardoor het vermogen om effectief te reageren op de vraag naar andere behoeften afneemt". 3. "Kippen passen zich aan koude stress aan door de hoeveelheid energie die wordt gebruikt voor groei en immuuncompetentie te beperken en de zo vrijgekomen energie te gebruiken voor thermoregulatie". 4. "Tussen groei, immuuncompetentie en thermoregulatie bestaan trade-offs".

Hoofdstuk 2 en 6 beschrijven de effecten van koude stress en beperkt voeren op verschillende componenten van het immuunsysteem en op de niveaus aan corticosteron in plasma in twee verschillende kippen lijnen met een verschillende genetische achtergrond. Over het algemeen had koude stress en beperkt voeren een stimulerend effect op de innate immuniteit, maar werd de specifieke humorale immuniteit niet beïnvloed (Tabel 1). Deze resultaten werden verder ondersteund door de expressie niveaus van verschillende cytokines in bloed cellen. (Hoofdstuk 7). Verder hadden de 2 stressoren koude stress en beperkt voeren een verschillend effect op de cel gebonden immuniteit en corticosteron niveaus. Koude stress had een stimulerend effect op de cel gebonden immuniteit, maar onderdrukte de corticosteron niveaus in plasma. Voedsel beperking resulteerde echter in tegengestelde effecten. De in hoofdstuk 3 en 5 beschreven correlatie tussen de veranderingen in plasma corticosteron niveaus en cel gebonden immuniteit suggereren dat de effecten van zowel koude stress als

voedsel beperking op de cel gebonden immuniteit worden bepaald door de concentratie aan corticosteron.

Het effect van verschillende perioden van koude stress op kippen met een verschillende genetische achtergrond worden beschreven in de hoofdstukken 2, 4 en 6 en de resultaten worden samengevat in tabel 1. De duur van de koude stress had tegengestelde effecten op verschillende componenten van het immuunsysteem. Alleen een lange blootstelling aan koude (1 week) liet een toename van de cel gebonden immuniteit zien (tabel 1) en reduceerde de toename in lichaamsgewicht in beide lijnen. Echter, de specifieke antilichaam reactie werd niet beïnvloed door de koude stress. De toegenomen cel gebonden immuunreacties correleerden met afgenomen corticosteron niveaus (tabel 1). Hogere niveaus aan triiodothyronine werden alleen gemeten in kippen die gedurende een korte tijd aan koude waren blootgesteld. Er werd echter geen correlatie met één van de gemeten immuunparameters waargenomen (hoofdstuk 3). De resultaten van deze experimenten laten duidelijk zien dat het effect van stress op het immuunsysteem afhankelijk is van de duur van de koude stress en van de gemeten immuunparameter.

	Immuunparameter								Endocriene parameter	
	NAb		ROI		SAb		CMI		CORT	
Tijdsduur	KS	BV	KS	BV	KS	BV	KS	BV	KS	BV
Kort (<1 wk)	↑	-	↓ / ↑	-	NS	-	↓ / ↑	-	↓	-
Lang (1 wk)	NS	-	NS	-	NS	-	↑	-	↓	-
Zeer lang (> 1 wk)	↓H / ↑L	↓	↓H / ↑L	↑	NS	NS	↑H / ↓L*	↓	-	↑

Tabel 1. Samenvatting van de effecten van koude stress (10 °C; KS) en beperkt voeren (60% *ad libitum;* BV) op verschillende componenten van het immuunsysteem, zoals a) Innate humorale Immuniteit (NAb, Natuurlijke Antilichaam Niveaus), b) Innate Cellulaire Immuniteit (ROI, reactieve zuurstof radicalen), c) Specifieke Humorale Immuniteit (SAb) and d) Cel gebonden Immuniteit (CMI) en de endocriene factor corticosteron (CORT). Alle experimenten werden uitgevoerd met gebruikmaking van kippen die divergent waren geselecteerd voor hoge- (H) of lage (L) immuunreactiviteit tegen een antigeen. De gedetailleerde experimentele opzet, methoden en de effecten zijn beschreven in de hoofdstukken 2-6. ↑ = significante toename, ↓ = significante afname, NS = niet significant effect, - = niet bepaald, * = effect van koude stress op beide lijnen was niet statistisch significant. De effecten werden als significant beschouwd als $P<0.05$.

In hoofdstuk 4 staat beschreven welke effecten koude stress heeft op diverse immuuncomponenten, waarbij de effecten zijn gemeten op verschillende tijdstippen na het beëindigen van de koude stress. De effecten van de koude stress verschilden niet van de observaties die hierboven zijn beschreven. In dit experiment werden stimulerende effecten van koude stress (1 week) op de cel gebonden immuniteit pas 21 dagen na de koude stress waargenomen. Dit laat het belang zien van het relatieve tijdstip van de meting van deze immuunparameter ten opzichte van de koude stress. Deze effecten werden echter niet waargenomen voor de andere gemeten componenten van het immuunsysteem.

De samenvatting van de resultaten in de vorige paragrafen laat zien dat koude stress en beperkt voeren tegengestelde effecten heeft op verschillende immuunfuncties, maar de effecten van de beide stressoren verschilden niet tussen de 2 selectielijnen (hoofdstukken 2, 4 en 6). Echter, de effecten van zeer lange blootstelling aan koude (> 1 week; zie hoofdstuk 7) op de innate immuniteit (natuurlijke antistoffen en reactieve zuurstof radicalen) en cel gebonden immuniteit (CMI) verschilde tussen de H en de L lijn (Tabel 1). Dit suggereert dat: 1. De effecten van de beide stressoren op de immuuncompetentie niet worden bepaald door de genetische achtergrond. 2. Genetische selectie voor een eigenschap (b.v. antilichaamproductie) geen invloed heeft op de fysiologische adaptieve capaciteit van kippen. Eén van de hypotheses waaraan in dit proefschrift aandacht is besteed (hoofdstukken 2, 4 en 6) was dat kippen zich aanpassen aan koude stress door zijn “resources” anders te verdelen over groei, het immuunsysteem en thermoregulatie. Als deze hypothese waar is, zou koude stress de immuuncompetentie en groei moeten hebben onderdrukt. De verwachte remmende effecten van koude stress werden echter alleen voor de groei waargenomen. In tegenstelling tot de verwachte afname van de immuuncompetentie namen zowel innate- als cel gebonden immuunfuncties toe. Dit zou kunnen betekenen dat kippen adapteren aan koude stress door een herverdeling van “resources” van groei naar thermoregulatie en immuunparameters.

In hoofdstuk 6 staat beschreven dat in H-lijn kippen blootstelling aan koude gedurende lange tijd de cel gebonden immuniteit wordt gestimuleerd en de innate immuniteit wordt geremd, terwijl in de L-lijn tegenovergestelde effecten werden gevonden (tabel 1). Deze resultaten geven een experimentele ondersteuning voor de mogelijkheid dat er een “trade-off”

bestaat tussen adaptieve en innate delen van het immuunsysteem. Verder lijkt deze "trade-off" te worden bepaald door de genetische achtergrond van het individu.

Belangrijke conclusies die op basis van de in dit proefschrift beschreven resultaten kunnen worden getrokken zijn:

- Koude stress en beperkt voeren hebben geen effect op de humorale specifieke immuniteit, maar stimuleren de innate immuniteit.
- Genetische selectie voor een gezondheidskenmerk zoals immuuncompetentie heeft mogelijk geen effect op tolerantie tegen gematigde stress en de fysiologische adaptieve capaciteit van kippen.

Een "trade-off"tussen thermoregulatie en immuunreactiviteit is niet aangetoond. De resultaten suggereren echter wel het bestaan van een "trade-off" tussen verschillende onderdelen van het immuunsysteem (adaptief en innaat).

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Basav
16th Jan 06
Wageningen

Curriculum Vitae

About the author

Basavarajappa Nagarajappa Hangalapura (Basav), born on 7th of May, 1977 in a small village called Hangalapura in Karanataka state, India. He obtained his B.F.Sc degree with high first class in 1999 from University of Agricultural Sciences, Bangalore, India. In the year 2000, he was offered a fellowship by NUFFIC under Netherlands Fellowship Program to pursue his M.Sc in Wageningen University. He obtained his M.Sc in Animal Science with first class in 2002 with a major thesis in Pathology, Virology and Epidemiology. Since 2002 till date he has been employed at the Dept of Animal Science, Wageningen University, as a junior researcher. The research carried out during that employment at Wageningen University has led to the current dissertation to obtain his PhD in 2006. Author carried out a part of the research presented in the current dissertation at S.J. Lamont's Lab, Dept. of Animal Science, ISU, IA, USA, in 2004.

List of Publications

Peer reviewed scientific publications in international journals

1. **Hangalapura B. N.**, M. G. Kaiser, J. Van der Poel, H. K. Parmentier, and S. J. Lamont (2005). Cold stress equally enhances pro-inflammatory cytokine gene expression in chicken lines divergently selected for antibody responses. **Dev. Comp. Immunol.** (Epub ahead of print).
2. **Hangalapura B. N.**, M. G. B. Nieuwland, G. De Vries 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(10):1520–9.
3. **Hangalapura B. N.**, M. G. B. Nieuwland, J. Buyse, B. Kemp and H. K. Parmentier (2004). Effect of duration of cold stress on plasma adrenal and thyroid hormone levels and immune responses in chicken lines divergently selected for antibody responses. **Poult. Sci.** 2004 Oct; 83(10):1644-9.
4. Parmentier H. K., W. J. A. Van Den Kieboom, M. G. B. Nieuwland, G. De Vries Reilingh, **B. N. Hangalapura**, H. F. J. Savelkoul, and A. Lammers (2004). Differential effects of lipopolysaccharide and lipoteichoic acid on the primary antibody response to keyhole limpet hemocyanin of chickens selected for high or low antibody responses to sheep red blood cells. **Poult. Sci.** 83(7) 1133-39.
5. Van Loon D. P. R., **B. Hangalapura**, G. De Vries Reilingh. M. G. B. Nieuwland, B. Kemp, and H. K. Parmentier (2004). Effect of three different housing systems on immune responses and body weight of chicken lines divergently selected for antibody responses to sheep red blood cells. **Live. Prod. Sci.** 85(2-3) 139-150.
6. **Hangalapura B. N.**, M. G. B. Nieuwland, G. De Vries Reilingh, H. Van Den Brand, B. Kemp, and H. K. Parmentier (2004). Durations of cold stress modulates overall

immunity of chicken lines divergently selected for antibody responses. **Poult. Sci.** 83 (5). 765-775. Featured article in **J. Exp. Biol.** 207 (21).

7. **Hangalapura B. N.**, M. G. B. Nieuwland, G. De Vries Reilingh, M. J. W. Heetkamp, H. Van Den Brand, B. Kemp, and H. K. Parmentier (2003). Effects of cold stress on immune responses and body weight of chicken lines divergently selected for antibody responses to sheep red blood cells. **Poult. Sci.** 82(11) 1692-700.

Peer reviewed scientific publications in national journals

1. Mathew M, **Basavarajappa H. N.** Gouda G and Gupta TRC (2001). Seasonal and temporal variation of Chlorophyll-a and Phaeopigments v/s nutrients and physcico-chemical parameters in Netravati - Gurpur estuary, Mangalore. Ind. J. Env. Sci.

Books, or contributions to books

1. Mohan, C. V., **Basavarajappa H. N.** (2001). Thematic review on management strategies for major diseases in shrimp aquaculture in India. Ohne Ort, S. 51-58. In: Rohana P. Subasinghe / J. Richard Arthur/MichaelJ. Phillips / Melba B. Reantaso (Hrsg.):
http://www.enaca.org/Shrimp/Case/Global/CEBU/FinalCEBU.pdf

Titles of popular articles, thesis and papers published in conference proceedings

1. **Hangalapura B.** (2005). Genetic background of an individual determines the sensitivity of innate component of humoral immunity for a novel environmental stressor. Proceedings of Australian Poultry Science Symposium, Vol. 17, 129-131.
2. **Hangalapura B.** (2004). Does cold stress affects immune responses via endocrine mediators? Proceedings of World Poultry Congress (Digital publication).
3. **Basavarajappa H. N.** (2002). A study on Eel viral diseases (HVA and EVE) - Test development, pathogenesis and epidemiology. MSc thesis. Wageningen UR, S20, No 1629.
4. **Basavarajappa H. N.** Gangadhar Gowda, and Iqlas Ahmed. (2000). Mussel farming for food security needs in developing countries. Info-Fish International, Issue 1.

Scientific publications in review or preparation

1. **Hangalapura B. N.**, B. Kemp, and H. K. Parmentier (2005). Genetic background determines the trade-off between the arms of immune system in domestic fowl *Gallus domesticus*. Proc. R. Soc. Lond. B (submitted).
2. **Hangalapura B. N.**, S. Bouwhuis, L. Star, B. Kemp, and H. K. Parmentier (2005). Exposure to low temperatures affects disease resistance against New castle disease in chickens. (in preparation).
3. **Hangalapura B. N.**, L. Star, S. Bouwhuis, B. Kemp, and H. K. Parmentier (2005). Fluctuating environmental temperatures positively affect immunity and body weight of chicken lines divergently selected for antibody responses. (in preparation).
4. **Hangalapura B. N**, P. W. van Tulden, and O. L. M. Haenen (2005). Pathogenesis of Herpes virus of European eel *Anguilla anguilla* (under preparation).

Abstracts published in conference / symposium proceedings

1. **Hangalapura B. N**, B. Kemp, and H. K. Parmentier (2005). Genetic background determines the trade-off between the arms of immune system in domestic fowl *Gallus domesticus.* Benulux Congress of Zoology. Wageningen, The Netherlands, October 26-28, 2005.
2. **H.N. Basavarajappa** and O.L.M. Haenen (2005). Pathogenesis of *Herpesvirus anguillae* (HVA) in juvenile European eel (*Anguilla anguilla*). European Association of Fish Pathologist Conference. Copenhagen, Denmark. September 11-16, 2005.
3. **Basavarajappa N. Hangalapura.** Genetic background of an individual determines the sensitivity of innate component of humoral immunity for a novel environmental stressor. Australian Poultry Science Symposium, Sydney, Australia, February 5-7, 2005.
4. **Basavarajappa N. Hangalapura**, M. G. B. Nieuwland, G. Reilingh, H. Brand, B. Kemp and H. K. Parmentier. Does hunger affects health? 8th Avian Immunology Research Group meeting, 2004, Munich, Germany.
5. **Basavarajappa N. Hangalapura**, M. G. B. Nieuwland, J. Buyse, B. Kemp and H. K. Parmentier. Does cold stress affects immune responses via endocrine mediators. World Poultry Congress, Istanbul, Turkey, June 8-13, 2004.
6. **Basavarajappa N. Hangalapura.** Feed restriction affects immune responses in chicken lines selected for antibody responses. WIAS science day 2004, Wageningen, NL, 2004.
7. **Basavarajappa N. Hangalapura**, M. G. B. Nieuwland, J. Buyse, B. Kemp and H. K. Parmentier. Cold stress suppresses corticosterone level and enhances cellular immune responses in chicken lines selected for antibody responses. WIAS Science Day 2004, Wageningen, NL.
8. **Basavarajappa N. Hangalapura**. Durations of cold stress modulates overall Immunity of chicken lines selected for antibody responses. NE-60 Annual meeting of genetic bases for resistance and immunity for avian diseases. University of Connecticut, USA, 2003.
9. **Basavarajappa N. Hangalapura**, M. G. B. Nieuwland, H. Van Den Brand, B. Kemp and H. K. Parmentier. Acute cold stress affects cellular but not humoral immune responses in chicken lines divergently selected for antibody responses. ISDCI 2003.
10. **Basavarajappa N. Hangalapura.** Effect of cold stress at different time points prior to immunization on immune responses in chicken lines selected for antibody responses. PhD retreat, "Gate way to the future", Nunspeet, NL, 2002.
11. **Basavarajappa N. Hangalapura.** Immune reactivity of poultry under non-steady state condition. NE-60 Annual meeting of genetic bases for resistance and immunity for avian diseases. Auburn university, USA, 2002.Haenen O. L. M., Van Tulden P. W, Dijkstra SG, Davidse A, Wagenaar F, Botter A,
12. **Basavarajappa H. N.** and Van Nieuwstadt A. P. Viruses isolated from European eel *Anguilla anguilla* in the Netherlands from 1986-2001.
13. **Basavarajappa H. N.** The Causal Web for White Spot Disease of Shrimp. Aquaculture Canada, Halifax, 2001.
14. **Basavarajappa H. N.,** Gangadhar Gouda. Seasonal distribution of nutrients in relation to salinity and temperature in Nethravati - Gurpur estuary, Mangalore. The Fifth Asian Fisheries Forum 2000, Orissa, India.

Training and Supervision Plan

Training and Supervision Plan	**Graduate School WIAS**	
*Name:*B.N.Hangalapura *Group:* Adaptation Physiology *Period:* 1st March 2002 - 1st March 2006 *Daily supervisor(s):* Dr.Ir. H. K. Parmentier & Dr.Ir. H. van den Brand *Supervisor:* Prof. Dr. Ir. B. Kemp		
The Basic Package	Year	ECTS
WIAS Introduction Course	2003	1.5
Course on Philosophy of Science and Ethics	2003	1.5
International conferences		
Genetic Bases for Resistance & Immunity to Avian Diseases, Auburn, USA	2002	0.9
Developmental and Comparative Immunology Congress, St Andrews, UK	2003	1.5
Genetic Bases for Resistance & Immunity to Avian Diseases, Connecticut, USA	2003	0.9
European Poultry Genetics Symposium, Wageningen, NL	2003	0.9
World Poultry Congress, Istanbul, Turkey	2004	1.7
Avian Immunology Research Group, Munich, Germany	2004	1.2
Australian Poultry Science Symposium, Sydney, Australia	2005	0.9
Seminars and workshops		
WIAS Science day 2002, 2003, 2004	2002-4	1.0
Phd Retreat 2002, 2004	2002	1.3
Seminar on Trypanosomiasis, Wageningen, NL	2002	0.5
Selection of Chickens; an approach to unravel immune competence	2005	0.2
Presentations		
Genetic Bases for Resistance & Immunity to Avian Diseases, USA (Oral)	2002	1.0
Phd Retreat 2002 (Oral)	2002	1.0
Developmental and Comparative Immunology Congress, St Andrews, UK (Poster)	2003	1.0
Genetic Bases for Resistance & Immunity to Avian Diseases, USA (Oral)	2003	1.0
WIAS Science Day 2004 (Oral and Poster)	2004	2.0
World Poultry Congress 2004 (Oral & Poster)	2004	2.0
Avian Immunology Research Group, Munich, Germany (Poster)	2004	1.0
Australian Poultry Science Symposium (Oral)	2005	1.0
In-Depth Studies		
Post Graduate Immunology course	2004	1.5
A Course on Bioinformatics and Genomics	2004	2.7
RNAi Technology and Application in Viral Disease	2004	0.6
Immunotechnology	2005	6.0
WIAS Advanced Statistics Course: Experimental Design	2005	1.0
Professional Skills (support courses)		
WIAS Course Techniques for Scientific Writing	2002	1.2
Use of Laboratory Animals	2002	4.5
Laboratory Use of Isotopes	2002	1.3

Training and Supervision Plan	Graduate School WIAS	
Research Skills (training)		
Carried Out Scientific Research at Iowa State University for 3 months, Ames,USA	2004	2.0
Teaching and Supervising Skills		
Adaptation Physiology I	2004	0.1
Supervised Three Graduate Students for their MSc Thesis	2002-5	5.5
Management Skills (training)		
Organized PhD Retreat-2004	2003	1.7
WAPS Council Member and Theme Team Representative	2003-5	3.0
TOTAL		**55.0**

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