Robustness in laying hens

Influence of genetic background, environment & early-life experiences

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Laura Star

Proefschrift

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Abstract

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Aim of the project 'The genetics of robustness in laying hens' was to investigate nature and regulation of robustness in laying hens and the possibility to increase robustness by breeding. A robust animal was defined as 'an animal that has the potential to keep functioning and take short periods to recover under varying environmental conditions'. The experiments described in this thesis investigated parameters of interest for robustness in laying hens, where the influence of genetic background, environmental conditions, and early-life experiences was used as framework. The first experiment aimed at genetic differences in innate (natural) humoral immune components between 12 purebred layer lines. Levels of innate and specific humoral immune competence depended on genotype. Within layer lines, however, natural antibodies (NAb), as part of innate immune competence, were related with survival, suggesting that levels of (NAb) are related with health. The second experiment aimed at influence of, or response to, environmental conditions, i.e., climatic stress (high temperature) and microbial challenge (lipopoly-saccharide). Comparable response patterns to climatic stress and microbial challenge were found within lines, but lines differed in response levels towards these stressors. The third experiment aimed at improvement of robustness by early-life experiences to climatic stress and microbial challenge. The data, however, did not reveal improvement of robustness by early-life experiences, apart from immune responses to the microbial challenge. Overall, the present studies indicate that robustness mainly depends on genetic background and environmental circumstances and to a minor extent on early-life experiences. The basic elements for robustness are survival, reproduction, and responsiveness towards environmental stressors, where a robust laying hen is a hen with a high survival rate, high production level, and low responsiveness towards environmental stressors. We have established a predictive value of the level of NAb for survival of the laying period of laying hens. Besides, NAb have a moderate heritability, giving opportunity for selection towards this trait. A principal component analysis indicated that performance parameters and innate immune parameters are most likely not related and selection on innate immune parameters will probably not be on the expense of hen-day egg production. Implementation of selection for NAb into a breeding goal might, therefore, improve robustness of laying hens.

Voorwoord

Vier jaar geleden begon ik aan het project ‘De genetica van robuustheid in leghennen’. Met name de term robuustheid sprak mij hierin aan; een breed en vaag begrip waarmee je alle kanten op kon. Maar dat maakte het ook lastig, want wat is robuustheid eigenlijk, hoe kan je het definiëren? En waar ga je beginnen, wat zijn interessante parameters die iets zeggen over robuustheid? Gelukkig waren er vele mensen die met mij mee wilden denken en werken, en die wil ik bij deze bedanken.

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CHAPTER 1

General introduction

INTRODUCTION

Until recently, animal breeding was mainly directed at improving production and less attention was paid at increasing health and welfare, although it was, and it is still, broadly recognized that health and welfare of production animals is important. Also in poultry, problems occur which are related to health and welfare. For instance, feather pecking (pulling out the feathers of pen mates), cannibalism and mortality due to diseases. These problems are of major concern in all types of poultry production systems. Furthermore, there is only a limited number of internationally operating poultry breeding companies, which have to provide laying hens worldwide. As a consequence, these companies face a wide variety of environmental conditions in which their laying hens have to perform (Knap, 2005), like different climates and different housing systems. For these companies it is favorable to have laying hens that can function under a wide variety of environmental conditions, without affecting health and welfare of laying hens.

In association with one of the few breeding companies for laying hens, Hendrix Genetics, the project 'The genetics of robustness in laying hens' was started. Hendrix Genetics formulated four priorities for what a robust laying hen needs:

- do not peck with intact beaks
- keep functioning/producing at high temperatures
- keep functioning/producing at high disease challenge
- keep functioning/producing with changing feed quality

The first priority is based on the fact that, for instance in the Netherlands, beak-trimming of laying hens will become forbidden within a couple of years. The incidence of severe feather pecking and cannibalism will increase when laying hens are not debeaked. Therefore, Hendrix Genetics likes to breed a laying hen with an intact beak that does not show severe feather pecking. The other three priorities are related to the fact that laying hens are shipped globally and have to face different circumstances. First, the poultry industry is moving to tropical and semi-tropical countries, where hens have to face high temperatures. Hendrix Genetics likes to have laying hens that perform well under warmer conditions, i.e., are able to maintain a high hen-day egg production. Second, higher temperatures, but also different housing systems, for instance organic housing, may influence hygienic conditions. Incidence of diseases may spread when hygienic conditions are low. Third, quality and quantity of feed will differ. For instance, laying hens are usually

fed a wheat-soy-based diet in the Netherlands, whereas in the USA laying hens are fed a corn-soy-based diet. Based on these facts, Hendrix Genetics realized that, to satisfy the markets in the 21th century, they must ensure an appropriate level of performance, health, and welfare of their laying hens, e.g., robust laying hens are required for future poultry farming.

ROBUSTNESS

At the start of the project 'The genetics of robustness in laying hens', robustness seemed to be a very general term that first had to be defined. Keeping in mind the four priorities formulated by Hendrix Genetics, robustness was defined as 'the potential to keep functioning and take short periods to recover under varying environmental circumstances'. Next step was to select parameters or traits that could give an indication of robustness, and that could be implemented into a breeding goal for robustness. At that moment there were no parameters or traits that described robustness.

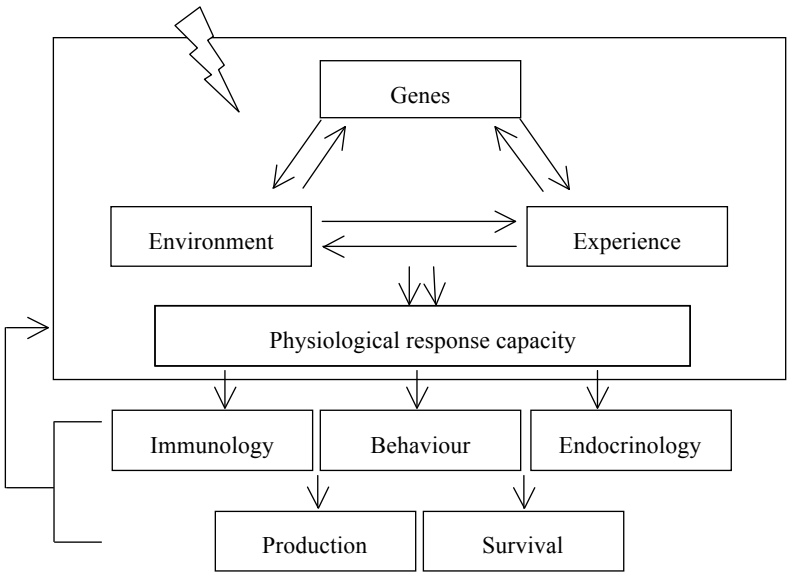

FIGURE 1.1. Robustness of an animal is influenced by genetic background, environment, and early-life experiences, where production, survival, behaviour, immune, and endocrine parameters can be used as read-out for robustness.

From a biological/evolutionary point-of-view, but also from a breeding point-of-view, survival and reproduction are the basic elements during the life-time of an individual.

Therefore, survival and (re)production are chosen as the core parameters for robustness. Besides, in order to survive and optimize (re)production, one of the most critical functions within an individual's life cycle is to respond to unpredictable events in the environment with appropriate behavioral and physiological adjustments (Wingfield and Kitaysky, 2002). Changes or fluctuations in behavioral and physiological adjustments illustrate the physiological response capacity of an individual under less predictable or unpredictable conditions. The responsiveness to unpredictable conditions comprises a complex, integrated system designed to prepare the individual for challenge or threat. The dynamics in this complex response system are shaped by heritable variation, along with environmental elements and developmental experiences (Figure 1.1). Reverting to the given definition of robustness, 'functioning' is a read-out for this complex response system, and can therefore be evaluated in terms of behavioral, physiological, and immunological performance.

ROBUSTNESS TRAITS

Ultimate goal of the project is to implement individual traits of a laying hen into a breeding goal for robustness. To utilize robustness as a breeding goal traits have to be, a) relevant, i.e., they have to say something about robustness, b) simple, i.e., they have to be understandable for users, c) reliable, d) it must be possible to establish a target value or trend, and e) data have to be accessible. Besides, interesting traits for robustness are assumed to be sensitive to unpredictable fluctuations in the environment, and need an immediate response in order to minimize stress and to maintain production. These traits cover immune, physiological, and behavioral characters. In this thesis a broad spectrum of physiological traits will come up; from body weight and hen-day egg production to endocrine parameters as corticosterone and 3,5,3'-triiodothyronine (T3). The focus will be, however, on innate humoral immune parameters as the first line of defense to invading pathogens.

Innate immunity

The environment contains a broad variety of infectious microbes, like viruses, bacteria, fungi, protozoa, and eukaryotic parasites. These can cause illness and will kill the host when they can multiply without any control. Most infections are, however, short-lived and cause less or no permanent damage. This can be attributed to the immune system that defends the body against infectious microbes. Immune defence is based on a variety of cell types with specific function. The two main types of responses are cellular and humoral responses (Schmid-Hempel, 2003). The humoral immune response can, on its turn, be

divided in specific and innate responses. The specific immune response has a memory function and therefore responds to specific targets. The innate immune response has no memory function and is a non-specific defence reaction. Because the innate immune system is non-specific it responds to every invading microbe, and acts as the first line of defence.

Furthermore, the innate immune system seems to be more sensitive to changing environmental conditions, than the specific immune system. For instance, innate immune responses were immediately enhanced when laying hens were exposed to cold stress, whereas the specific immune responses did not change or just after an prolonged period (Hangalapura et al., 2003,2004a,b). Because the innate immune system seems to be more sensitive to changing environmental conditions, this part of the immune system is of more interest for robustness than the specific immune system. In addition, laying hens receive a large number of vaccinations in the first 16 weeks of their life. Therefore, the specific immune system of laying hens is well prepared for the most common diseases in poultry farming. Still, a number of laying hens will not survive the end of lay, and die of non-specific causes. Ochsenbein and Zinkernagel (2000) argued that non-specific (innate) immunity might play an important role in enhancing survival of the host by providing early resistance against infection.

Related to this, the innate immune system can be divided in several components: cells (granulocytes, monocytes, macrophages, and dendritic cells and natural killer cells) next to humoral components such as acute phase proteins, natural antibodies, and complement. Both natural antibodies (NAb) and complement can be easily measured on relatively large scale, which makes them of interest as parameter for robustness. Besides, both components are highly important in initiation of specific immune responses. An ineffective level and low diversity of natural antibodies is, for instance, a serious risk factor for health and survival, because an ineffective level or low diversity of natural antibodies causes a delayed specific immune response resulting in increased influenza induced mortality (Baumgarth et al., 1999; Harada et al., 2003; Jayasekera et al., 2007).

Finally, traits have to be implemented in the breeding goal of robustness. Siwek et al. (2006) already estimated a heritability of NAb binding to lipopolysaccharide (LPS) of 0.17 and 0.09 at 5 and 18 wk of age, respectively, in a layer chicken population selected for specific antibody response to sheep red blood cells (SRBC), and a heritability of 0.23 at 38 wk of age in a layer chicken population selected for production traits. This indicates that breeding for natural antibodies in possible. Therefore, typing and selecting for such innate immune responses might be a sensible approach for characterization and subsequently improving robustness in laying hens.

NATURE OF ENVIRONMENTAL STRESSORS

As mentioned before, one of the most critical functions within an individual's life cycle is to respond to or to cope with unpredictable events in the environment with appropriate behavioral and physiological adjustments (Wingfield and Kitaysky, 2002). Unpredictable events in the environment that could be related to the four priorities given by Hendrix Genetics are changing temperatures and hygienic circumstances.

Because the poultry industry is moving towards more tropical countries we have chosen high temperature challenge (32ºC) as stressor. Heat stress is a powerful stressor which disturbs the physiology of the body. Already in 1979, Mount reviewed the effects of changing thermal environments on production animals. When the ambient temperature is below 30ºC the bird loses heat from the body through physiological mechanisms such as conduction, convection, and radiation. Also egg production can be maintained when temperature is below 30ºC. Above 30ºC the physiological mechanism becomes inefficient so the bird resorts to panting. Feed intake of birds fed *ad libitum* falls as temperature rises. The depression in egg production brought about by high temperatures is probably due to the reduction in feed intake. Furthermore, high temperature reduces egg weight and egg shell thickness.

The level of microbiota in the environment contributes to the hygienic circumstances. A major poultry health problem is caused by inhalation of environmental gram-negative bacteria (in particular their endotoxin; Zucker et al., 2000). These gram-negative bacteria are ubiquitous in the environment of poultry (Chapman et al., 2005). Endotoxins may affect the type and magnitude of immune responses in poultry, which is of major importance for vaccination and health strategies (Maldonado et al., 2005). Lipopolysaccharide (LPS), derived from intestinal gram-negative microbiota, is such an endotoxin, and is often used as a model antigen to study the animal's susceptibility to (non-specific components of microbiological) pathogens and capability to adapt to immune stressors. Administration of LPS causes sickness symptoms including changes in body temperature (fever), reduced body weight gain, and changes in behaviour (Adler and DaMassa, 1978; Cheng et al., 2004; Macari et al., 1993; Xie et al., 2000), but also involves immune stimulation, including modulation of antibody responses (Gross and Siegel, 1975; Maldonado et al., 2005; Parmentier et al., 1998,2004c), probably by activation of innate immune responses.

Both heat exposure and microbial challenges (LPS) are common in poultry farming and, therefore, it is conceivable that interactions between heat exposure and microbial challenges occur. Understanding of the interactions between these two stressors is of interest, because they represent two physiological drives that utilize common effectors but

may induce opposite responses (Blatteis, 2000). During heat exposure a rise in body temperature is prevented by panting and vasodilatation of the skin, whereas a microbial challenge (LPS) is associated with a rise in body temperature (fever) which is achieved by increased metabolic heat production and vasoconstriction of the skin.

We have chosen for high temperature and a microbial challenge as stressors, but, in principle, any external stimulus that challenges homeostasis can be viewed as a stressor, and the changes in biological function, e.g., physiological response capacity (Figure 1.1), that occur as the animal attempts to maintain homeostasis constitutes the animal's stress response (Moberg, 1985). The responses, as will be measured in physiological, immune, and endocrine parameters, will vary within and between chicken lines and will depend on the type of stressor, e.g., heat or microbial challenge. These differences in the perception of a stressor and in the nature of the biological response (sensitivity) may reflect the robustness of an animal.

AIM AND OUTLINE OF THE THESIS

The aim of the project 'The genetics of robustness in laying hens' is to investigate nature and regulation of robustness in laying hens under sub-optimal conditions and the possibility to increase robustness by using animal breeding without loss of production. Hereby the following questions need to be answered: How can robustness of laying hens be measured? Is there genetic variation for robustness parameters? Is it possible to develop a breeding program that improves robustness while maintaining production? The experiments described in this thesis investigated parameters that could predict the robustness of purebred layer lines, where the influence of genetic background, environmental conditions, and early-life experiences was used as framework (Figure 1.1). However, first the concept of robustness, the relation between the concept of robustness and the concepts of health, welfare, and integrity, and the implementation of the concept of robustness into a breeding goal will be described in Chapter 2.

The experiments for investigation of parameters for robustness based on the framework of genetic background, environmental conditions, and early-life experiences will be described in Chapter 3 to 7. The experiment described in Chapter 3 aimed at genetic differences in innate (natural) humoral immune components between 12 purebred layer lines and the relation between natural antibodies and survival. The experiments described in Chapter 4 to 6 aimed at influence of or response to environmental conditions, i.e., high temperature and microbial challenge. The four purebred layer lines used in these studies were characterized by levels of innate immune competence and survival rate as described in

Chapter 3. Chapter 4 describes effects of single or combined environmental stress on innate and specific humoral immune competence. Chapter 5 describes effects of single or combined environmental stressors on performance parameters (e.g., feed intake, body weight, hen-day egg production). Chapter 6 describes effects of single or combined environmental stressors on endocrine and oxidative stress responses. The experiment described in Chapter 7 aimed at investigating effects of early-life experiences with environmental stressors (high temperature and microbial challenge) in one layer line characterized by the studies described in Chapter 4 to 6.

The major findings of the experiments will be briefly discussed in the General Discussion (Chapter 8). The focus of the General Discussion, however, will be on robustness. How are the results of the experiments related to robustness? How can robustness be indicated on line level and on individual level? Which parameters are of interest for robustness, and could these parameters be implemented into a breeding goal for robustness?

CHAPTER 2

A PLEA TO IMPLEMENT ROBUSTNESS INTO A BREEDING GOAL: POULTRY AS AN EXAMPLE

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ABSTRACT

The combination of breeding for increased production and the intensification of housing conditions have resulted in increased occurrence of behavioral, physiological, and immunological disorders. These disorders affect health and welfare of production animals negatively. For future livestock systems, it is important to consider how to manage and breed production animals. In this paper, we will focus on selective breeding of laying hens. Selective breeding should not only be defined in terms of production, but should also include traits related to animal health and welfare. For this we like to introduce the concept of robustness. The concept of robustness includes individual traits of an animal, which are relevant for health and welfare. Improving robustness by selective breeding will increase (or restore) the ability of animals to interact successfully with the environment and thereby to make them more able to adapt to an appropriate husbandry system. Application of robustness into a breeding goal will result in animals with improved health and welfare without affecting the integrity. Therefore, in order to be ethical acceptable, selective breeding in animal production should accept robustness as a breeding goal.

Key words: health, integrity, laying hen, robustness as a breeding goal, welfare

INTRODUCTION

There is only a limited number of internationally operating poultry breeding companies that have to provide laying hens worldwide. As a consequence, these companies face a wide variety of environmental conditions in which their laying hens have to perform (Knap, 2005). Differences in environmental conditions can be due to climate, housing facilities, disease pressure, exposure to different pathogens, and differences in feed quality and composition. Laying hens are kept from the cold, dry climates in Siberia to the hot, humid climates in Brazil, from battery cages to free range systems that could differ in hygienic circumstances, and are fed corn-based to soy-based diets. Laying hens kept under such different conditions must be able to cope with their environment and, therefore, require sufficient capacities to adapt. Furthermore, these laying hens are expected to produce a maximum number of eggs irrespective of environmental circumstances.

Traditional breeding has resulted in a rapid increase in egg production; in 1930 the average production was 116 eggs per hen per year, whereas nowadays the average production is increased to 300 eggs per hen per year (Preisinger and Flock, 2000). Furthermore, production became even more efficient by intensification; farms increased in size and animals were kept at a higher density (Sandøe et al., 2003).

The combination of breeding for increased production and the intensification of housing conditions have not been without consequences, especially for the animals. Laying hens have become more at risk for behavioral, physiological, and immunological disorders (Rauw et al., 1998) and consequently, for reduced animal welfare. Behavioral disorders include cannibalism,[1] feather pecking,[2] and absence of broodiness behavior[3] (Newberry, 2004; Price, 1999; Savory, 1995); physiological disorders include asymmetric growth[4]

[1] Cannibalism is the act of consuming tissue of other members of the same species, whether living or dead, and at any stage of the life cycle. Cannibalistic behaviour affects the well-being of attacked laying hens, as evidenced by injuries which, if extensive, result in death (Newberry, 2004).

[2] Feather pecking is characterized as non aggressive pecks towards the plumage of other birds. Generally two forms can be distinguished, i.e., gentle and severe feather pecking. Gentle feather pecking can be defined as repeated pecks at the tips and edges of feathers, mostly ignored by the receiver. Severe feather pecking causes feather damage and feather loss. Flocks with high incidence of severe feather pecking suffer from reduced welfare and higher mortality rates due to cannibalism (Savory, 1995).

[3] Broodiness behavior consists of termination of egg production, the incubation of eggs, and care of the young (Johnson, 2000).

[4] Fluctuating asymmetry is defined as small, randomly directed deviations from perfect symmetrical development in bilateral traits, resulting from the inability of individuals to undergo identical development on both sides of the plane of symmetry. Fluctuating asymmetry provides a useful measure of how well development processes cope with internal genetic and external environmental stressors during morphogenesis (Tuyttens, 2003).

(Tuyttens, 2003; Yngvesson and Keeling, 2001) and osteoporosis[5] (Bishop et al., 2000; Whitehead et al., 2003); and immunological disorders include increased susceptibility against Marek's disease[6] (Dalgaard et al., 2003).

The traditional strategy to reduce these problems is preventive management. Preventive management can be divided in two procedures; physical and non-physical. A physical procedure to reduce feather pecking and cannibalism is beak trimming (Appleby et al., 2004) and non-physical procedures include decrease of light intensity, change of feed composition, environmental enrichment, and optimizing group size (Hester, 2005). To protect against harmful pathogens, vaccination can be used as a physical procedure, whereas high hygiene systems [specific pathogen free systems (SPF)] are used as a non-physical proceeding. Although much research has been focused on improvement of management factors, problems still occur in all types of poultry production systems. Furthermore, management factors used to reduce feather pecking and cannibalism, such as beak trimming and low light intensity, have been associated with welfare problems (Gentle, 1986; Jones and Hocking, 1999; Manser, 1996). Because of these welfare problems, beak trimming is, or will be in the near future, prohibited in parts of Western Europe.

Besides the traditional strategy of preventive management, another possibility is to adapt animals by selective breeding or even genetic modification. Selective breeding can be used to improve health and welfare related traits in laying hens (Jones and Hocking, 1999). Health can be enhanced by selective breeding for disease resistance. This may be effective in resistance to a wide range of pathogens and can be used to protect laying hens under different environmental conditions (Lamont, 1998). Welfare can be enhanced by selection against expression of undesirable behavior. Jones and Hocking (1999) argued that selection against feather pecking and cannibalism might provide powerful, welfare-friendly solutions.

Improving health and welfare by adapting the animal to the housing system, however, can result in violation of the integrity of the animal; for instance, breeding blind laying hens. It is technically possible to breed blind laying hens, which do not show feather pecking or cannibalistic behavior. Although these laying hens are blind, they are healthy, able to find food and water, and produce a number of eggs according to the expectations (Ali and Cheng, 1985). These hens also seem well adapted to their situation and, assuming

[5] Osteoporosis in laying hens is defined as a decrease in the amount of fully mineralized structural bone, leading to increased fragility and susceptibility to fracture (Whitehead et al., 2003).
[6] Marek's disease is caused by a highly virulent herpes virus. Marek's disease causes paralysis and mortality in laying hens (Bumstead, 2003).

that blind hens do not suffer in any other way, they may live a better life than hens that are able to see. Many people, however, intuitively feel that this is a morally wrong approach to improve animal welfare (Sandøe et al., 1999). In this example, integrity of the laying hens was violated by selective breeding. By making use of genetic modification, violation of the integrity could even be worse.

In present poultry farming, increased occurrence of behavioral, physiological, and immunological disorders affect health and welfare negatively. Preventive management and selective breeding to reduce disorders, like beak trimming or breeding blind laying hens, can affect the integrity of laying hens. For future livestock systems it is, therefore, important to consider how to manage and breed laying hens. In this paper, we will focus on selective breeding of laying hens. We argue that in future livestock systems it is necessary that breeding goals[7] should not only be defined in terms of production, but that they should also include traits related to animal health and welfare. For this we introduce robustness as a breeding goal.

Robustness is a term that is rapidly becoming a main interest in animal production (Knap, 2005; Ten Napel et al., 2006). We like to explore the discussion on robustness as a breeding goal for animals kept in future livestock systems. The concept of robustness is related to the concepts of health, welfare, and integrity, but in our opinion, robustness is more comprehensive. We expect that robustness as a breeding goal will result in better health and welfare without affecting the integrity of the laying hen. Based upon this, we argue that it is ethical acceptable to use selective breeding in order to create animals that are able to function better in conventional agricultural systems.

THE CONCEPT OF HEALTH, WELFARE, AND INTEGRITY

Before going into detail about the concept of robustness, the concepts of health, welfare, and integrity will be explored. For the concept of robustness it is important to have a perception about the definitions and considerations behind the realization of the concepts of health, welfare, and integrity. The considerations are important for the implementation of the different concepts into a breeding goal for robustness.

The concept of health

Different approaches towards the concept of health can be found in literature. The very basic definition of health is no more than the absence of disease (Gunnarsson, 2006;

[7] The definition of breeding goal will be elaborated in section 3.1.

Nordenfelt, 2007). Boorse (1997 in Nordenfelt, 2007) defined disease as 'a type of internal state that is an impairment of normal functional ability.' This definition indicates that disease (and health) are linked to functional ability, i.e., biological functioning (Nordenfelt, 2007). For Boorse (1997), biological functioning is tied to the individual's survival and reproduction. This is, however, a very narrow concept of biological functioning. The broader concept of biological functioning, as basis for the concept of health, is related to homeostasis, i.e., regulation of the internal environment of living organisms (Gunnarsson, 2006). In addition, an animal may be in pain and disabled by internal bodily causes (failure in regulating homeostasis) without reducing the probability of the animal's survival. This indicates that there are other possible goals than the one of pure survival (Nordenfelt, 2007). One goal related to health, and commonly used in the debate about animal welfare, is quality of life, which includes psychological aspects of health (Fraser et al., 1997). Gunnarsson (2006), however, mentioned that if health is defined as physical and psychological well-being, there will be problems associated with applying the definition to all animals, especially production animals. Gunnarsson (2006) stated that a health definition that puts priority to the physical and psychological well-being of a production animal is misleading in relation to the general purpose of livestock production. In livestock production, economical considerations are involved and can be decisive in the judgment of the animals' health. To achieve good health the animal has to be in harmony with itself and its environment, and has to be in a normal physical condition (free of diseases and other physical disorders) (Rutgers, 1993). Health could than be considered as "the physical condition required to achieve welfare at an acceptable level" (Brom, 1997 derived from Nordenfelt, 1987).

The concept of welfare

Welfare of farm animals is a major concern, in society, in livestock production, as well as in animal science (Kanis et al., 2004). Animal welfare, however, is a complex concept that is difficult to define operationally, and hence to evaluate empirically (Rowan, 1997). This has led to different welfare definitions.

Fraser et al. (1997) suggested that three main ideas are expressed in public discussion concerning animal welfare: feelings, functioning, and natural living. Fraser et al. (1997) also argued that a scientific approach to animal welfare has to take into account these ideas expressed in public discussion. Animal feelings are related to experiences of animals, i.e., mental harmony, whereas functioning is related to biological functioning, i.e., physical harmony. The concept of experience is based on the presence of positive experiences and

the absence of negative experiences, whereas the concept of functioning is based on "doing well," so that the animal is functioning as it should do (Stafleu et al., 1999). The idea that animals should live natural lives includes considerations of an animal's nature or telos (Appleby and Sandøe, 2002), which is related to the concept of integrity, and will be discussed later.

A definition of animal welfare related to the concept of experience is that 'animals should feel well by being free from prolonged and intense fear, pain, and other negative states, and by experiencing normal pleasures' (Fraser et al., 1997). Kanis et al. (2004) considered animal welfare as similar to 'animal happiness,' which can be seen as 'the balance between an animal's positive and negative emotions or feelings over a certain time period.' It is, however, impossible to ask an animal directly in which situation it feels comfortable and if its preferences are satisfied. Therefore, making use of the concept of experience in scientific studies is rather difficult. To make animal experiences more applicable, the concept of functioning can be used as a tool. The concept of functioning often involves ideas about evolutionary fitness, including successful breeding. When breeding is strongly affected by human intervention, as for production animals, it might be difficult to apply the concept of functioning (Appleby and Sandøe, 2002). The concept of functioning, however, can still be linked to scientific (biological, physiological, social functioning) animal production theories, or models. Definitions of welfare commonly used are often based on the concept of functioning. For instance, welfare definitions given by Broom (1993) 'welfare of an animal is reflected by the success of its attempt to cope with its environment' and by Siegel (1995) 'welfare depends on physiological ability to respond properly in order to maintain or re-establish homeostatic state or balance.'

For scientific models, the concept of functioning is easier to demonstrate than what an animal experiences (Duncan and Fraser, 1997). Although the concept of functioning is more straightforward to quantify, the link between (biological) functioning and the animal's welfare is not always apparent, e.g., there is little consensus on the baseline that should be used in assessing measures and there is less agreement on which levels necessarily denote a better quality of life for the animal. Therefore, assessment of welfare involves a mixture of scientific knowledge and value judgments.

The concept of integrity

Integrity has been described by Rutgers and Heeger (1999) as the 'wholeness and intactness of the animal and its species-specific balance, as well as the capacity to sustain itself in an environment suitable to the species.' The principle of respect for the integrity of animals

leads to considerations and arguments beyond animal health and welfare (Grommers et al., 1995). The integrity theory of King (2004) proposed that the value of animal life is such that animals should not be harmed or destroyed. The loss of life itself is conceived as the ultimate harm to the animal's integrity, i.e., to its 'completeness.'

Integrity gives notion to our own moral position, purposes, and perspectives with regard to animals (Vorstenbosch, 1993). Integrity is not a strictly describing term, but it rather refers to the way we think an animal has to be (Brom, 1997). In the former, we already mentioned the possibility to breed blind laying hens and that many people intuitively feel that this is a morally wrong approach to improve animal welfare. The moral notion that gives voice to this intuition is integrity (Bovenkerk et al., 2002). Another example is non-broody behavior in laying hens. Selection has resulted in strains of chickens that normally do not incubate eggs or brood chicks (Price, 1999). These laying hens seem to be well adapted to their situation and, probably, are still able to brood. However, they do not have the motivation to express their brooding behavior; it is just not natural to them. These two examples clearly show that it is important to consider the nature and biological needs of animals.

According to Rollin (1989), the nature and biological needs are related to the telos of an animal. He defined telos as 'the unique, evolutionarily determined, genetically encoded, environmentally shaped set of needs and interests which characterize the animal in question.' Each animal has a telos that is unique to its species, it can be seen as the 'chickenness of the chicken' or the 'pigness of the pig,' which are essential to their well-being as speech is to us (Rollin, 1989). He stated that the animal's well-being is determined by the match between its needs and interest and the treatment it receives (Rollin, 1995). Although, the animal's telos is unique to its species, Rollin (1995) argued that changing the telos of an animal can be justified. He stated that there is no moral problem in making an animal happier or prevent it from suffering by changing its telos, unless changes endanger the animal itself, other animals, humans, or the environment. Verhoog (1992), however, insisted that telos is of direct moral relevance in itself and should not be violated or changed. He stated that selective breeding is morally questionable, because it represents interference with the natural species integrity and evolutionary development of animals. De Vries (2006), however, stated that selective breeding cannot change the genes of animals, let alone introduce new genes. According to him, integrity is only violated if new genes are introduced to the genome of an animal and, therefore, selective breeding cannot violate the animals' integrity. In our opinion, this is too simple; selective breeding can violate the

animals' integrity in extreme cases like breeding blind laying hens. We can use selective breeding to improve animals, but only if the animals' identity is preserved.

THE CONCEPT OF ROBUSTNESS

Introduction to robustness

In the previous chapter we have explored the concepts of health, welfare, and integrity. All three concepts are related to the quality of life of an animal. To improve the quality of life of an animal in future livestock systems these concepts have to be integrated into a breeding goal. The breeding goal defines which traits have to be improved and how much weight is given to each trait. The breeding goal is the direction in which we want to improve the population (Cameron, 1997). The concepts of health and welfare primarily focus on the state of the animal (mentally and physically) in a specific situation. These concepts do not consider animal related traits and, therefore, could not be implemented into a breeding goal. Integrity considers animal related traits, namely the presence of species specific characters, e.g., it's 'completeness.' It is, however, not possible to optimize the integrity of an animal, and therefore integrity cannot be improved by selective breeding. For this, we would like to introduce the concept of robustness. The concept of robustness includes individual traits of an animal that are relevant for health, welfare, and integrity. Because robustness includes individual traits, it can be integrated into a breeding goal.

The concept of robustness is defined in different fields, e.g., ecology, biological systems, statistics, and animal production. A broad definition of the concept of biological robustness is 'the maintenance of specific functionalities of the system against perturbations, and it often requires the system to change its mode of operation in a flexible way' (Kitano, 2004). This definition can be used as a starting point for definitions of robustness in other fields, like animal production. Knap (2005) defined robust pigs as 'pigs that combine high production potential with resilience to external stressors, allowing for unproblematic expression of high production potential in a wide variety of environmental conditions.' Whereas Ten Napel et al. (2006) defined robustness in a broad sense as 'the minimal variation in a target feature following a disturbance, regardless of whether it is due to switching between underlying processes, insensitivity or quickly regaining the balance,' and in a narrow sense as 'the ability to switch between underlying processes to maintain balance.' The definitions of Ten Napel et al. (2006) are independent of species.

From these definitions, it can be concluded that the main characteristics informative for robustness of production animals are production and adaptation in a wide variety of

environmental conditions. Production is important because it is one of the parameters related to the functioning of an animal. Besides, production is important because of its economical value. In the concept of robustness, adaptation can be seen as a mechanism of the animal that enables it to cope with internal or external disturbances, or with changes in the environment. Ideally, we would like to breed a strain of laying hens that can adapt to different environmental conditions. In practice, however, strains of laying hens can perform differently in different environments; this is called genotype by environment interaction (Falconer and Mackay, 1996). As mentioned earlier, there is a limited number of internationally operating poultry breeding companies that provide laying hens worldwide. For these companies, it is favorable to have animals that can function under a wide variety of environmental conditions.

Using the main characteristics informative for robustness, e.g., production, adaptation, and a wide variety of environmental conditions, we define a robust laying hen as 'an animal under a normal physical condition that has the potential to keep functioning and take short periods to recover under varying environmental conditions.' Functioning can be evaluated in terms of physiological, behavioral, and immunological traits. This definition of robustness includes different measurable characteristics and traits that make the concept of robustness applicable for breeding programs.

Implementation of health in the breeding goal for robustness

In the definition of robustness, 'keep functioning' and 'take short periods to recover' are referring primarily to health. The definition of Rutgers (1993), 'the harmony between an animal itself and its environment, where the animal is free of diseases and other physical disorders,' primarily focuses on 'functioning.' Whereas the definition of Gunnarsson (2006) 'regulation of the internal environment of living organisms,' primarily focuses on 'take short periods to recover.' Robust animals will be less sensitive to disease pressure and are expected to recover more quickly than less robust animals. Therefore, by implementing the concept of robustness as a breeding goal, the health of laying hens should improve simultaneously.

Implementation of welfare in the breeding goal for robustness

Together, the welfare definitions given by Broom (1993) and Siegel (1995) 'welfare of an animal is reflected by the success of its attempt to cope with its environment' and 'welfare depends on physiological ability to respond properly in order to maintain or re-establish homeostatic state or balance,' respectively, corresponds with the definition of the concept

of robustness. The distinction between animal welfare and robustness is that animal welfare is often measured by an animals' response to a current stressor, whereas robustness is based on the possibility to respond adequately to a stressor and is aiming at less disturbed functioning by challenge with a stressor. Implementation of robustness into a breeding goal should result in animals with improved coping abilities for conventional housing systems, and, therefore, should result in improved animal welfare.

Implementation of integrity in the breeding goal for robustness

As described earlier, the concept of integrity indicates how an animal has to be. We have to be aware that selective breeding can have either positive or negative side effects on the ability to function. Sometimes a change in genotype would be an advantage to both animals and humans (Sandøe et al., 1999). But in other cases it could have a negative side effect. These negative side effects are not only morally problematic due to undesired consequences for health and welfare. They are also problematic because two core elements in the concept of integrity, as described by Rutgers and Heeger (1999) are at issue, namely 'the balance in species specifity' and 'to sustain itself in an environment suitable to the species.' According to Rollin (1995), changing the animal by selective breeding does not necessary lead to impoverishment of the telos. In line with this, notion of integrity is a requirement for robustness. Therefore, improvement of health and welfare by implementation of the breeding goal of robustness should not be achieved by violation of the integrity or impoverishment of the telos.

Application of robustness as a breeding goal

As mentioned earlier, robustness embraces health, welfare, and integrity. Therefore, different traits can be implemented in the breeding goal of robustness. To utilize robustness as a breeding goal, the traits have to be a) relevant, i.e., they have to say something about robustness, b) simple, i.e., they have to be understandable for users, c) sensitive, i.e., they have to react to changes in the system, d) reliable, i.e., different measurements must lead to the same outcome, e) it must be possible to establish a target value or trend, and f) data have to be accessible. Robustness as a breeding goal can be used for different production animals. Each production animal has its species specific characteristics. In this paper, we will focus on traits interesting for improvement of robustness in laying hens. An overview will be given of traits that can be implemented into a breeding goal. These traits cover

behavioral, physiological, and immune characters. In practical - commercial - context, selection for these robustness traits must be in balance with selection for production traits.

Traits to breed for

Behavioral traits. To quantify behavioral aspects for robustness in laying hens, parameters like fear, social stress, feather pecking, and cannibalism could be used. The different behavioral parameters are related. For instance, fearful laying hens tend to show more feather pecking behavior (Jones et al., 1995), and severe feather pecking can lead to cannibalism. Methods used to assess fear in laying hens involve fear towards humans or towards a novel object. Whereas determining plumage and skin condition is a method to assess feather pecking behavior. Variation in fearful behavior (novel object test) and incidence of feather pecking exists between genetically different layer lines (Uitdehaag et al., 2007). Rodenburg et al. (2004) estimated heritabilities for fearful behavior (open-field test) and feather pecking behavior ranging between 0.35 and 0.60, and 0.10 and 0.24, respectively. The estimated heritabilities were based on individual measurements. More or less fearful and pecking behavior, however, will also depend on the social behavior of group members, e.g., plumage condition of a hen does not only depend on her own pecking behavior, but also depends on the pecking behavior of her group members. Therefore, it is important to use a breeding method that makes use of information of group members, rather than individual information (Ellen et al., 2007; Muir, 2003).

Immunological traits. Animal health data are rarely straightforward to use. Veterinary treatment records do not give a precise measure for disease (Sørensen et al., 2001), and diagnoses do not normally describe implications useful for robustness. Increasing robustness of animals is important to reduce occurrence of diseases. To reduce occurrence of diseases, animals need a well developed immune systems that adequately responds to invading pathogens. The immunological capacity of animals might be enhanced by genetic selection for disease resistance. Variation in immune competence exists between genetically different layer lines (Star et al., 2007a). Siwek et al. (2006) estimated heritabilities for natural antibodies determined in blood ranging between 0.11 and 0.42, whereas Bovenhuis et al. (2002) estimated heritabilities for specific antibodies ranging between 0.16 and 0.19. Furthermore, immune responses towards environmental stressors vary between layer lines (Star et al., 2007b). Therefore, genetic selection for immune traits may improve resistance to a wide range of pathogens and may be an effective strategy to protect laying hens under a wide variety of environmental conditions (Lamont, 1998).

PHYSIOLOGICAL TRAITS. Genetic selection for production efficiency can have adverse effects on health. In poultry, for instance, this selection has unwittingly produced birds with poor structural bone mass (Bishop et al., 2000; Whitehead et al., 2003). Laying hens selected for high egg number and low maintenance requirements (which implies a small body mass) can become prone to osteoporosis towards the end of the laying cycle, because of the high metabolism of calcium for egg shell formation. Such birds have fragile bones and when caught and transported, fractures are common (Hughes and Curtis, 1997). Because selection for egg production has contributed to osteoporosis, this implies that susceptibility to osteoporosis has a genetic component. Bishop et al. (2000) found that traits describing bone strength are moderately to strongly inherited, where heritabilities range between 0.30 and 0.45. Therefore, selection for enhanced bone strength can be used to alleviate the problem of osteoporosis in laying hens.

POTENTIAL FOR A SUCCESSFUL RESULT

In our opinion robustness as a breeding goal can be successful to improve health and welfare of production animals in future livestock systems. Before robustness can be implemented into a breeding goal, large scale genetic research on the different traits has to be done. Large scale genetic research is for most traits labor intensive and expensive. For instance, behavioral measurements and collecting blood samples for immunological parameters have to be done at the individual level.

After determining the most promising traits, the next step will be the implementation of these traits into the breeding goal. Implementation of the traits is difficult and riskful, but the potential of success for robustness as a breeding goal depends on this implementation. One of the difficulties for the implementation is to decide which trait is more important than another, e.g., how much weight is given to each trait. It is, however, important to implement all traits, because the success of selective breeding for robustness depends on all traits and not on a single trait.

Genetic research for robustness traits and the implementation of these traits into the breeding goal have to be established by cooperation between science and breeders. Additionally, successful result of robustness as a breeding goal depends on the opinion and motivation of the farmer. The principle aspects of robustness may be different for each individual farmer (or breeder), but also reference values can change. Besides, in the future, other traits may arise that have to be implemented into the breeding goal of robustness. By implementation of new traits, it is, however, important that these traits concern the animal itself.

Finally, the potential for a successful result of robustness as a breeding goal depends on the economic value. In his decision-making, a farmer has to consider not just animal robustness, but also how to produce efficiently, at competitive cost.

QUESTIONS RELATED TO ROBUSTNESS

In this paper, we explored the discussion of robustness as a breeding goal for laying hens kept in future livestock systems. Although we think it is possible to implement robustness into a breeding goal, it still raises several ethical questions like: Is it acceptable to adapt animals to the production environment, rather than by changing their environments? Should animals be adapted to all environments, even the worst? And does selection for robustness affect the integrity of the animal?

When looking at the definition of robustness, a robust animal is an animal that has the potential to keep functioning and take short periods to recover under varying environmental conditions. This indicates that the animal has to function under a wide range of circumstances. It is, therefore, preferable to select for robustness traits that are common to different types of production environments. But, are we really aiming at adapting the animal to even the worst environment? No. The aim is to breed animals that can function well in a range of environments and not to breed animals specifically for the worst environments. However, even in the most optimal environments welfare of laying hens can be improved as illustrated by the fact that they show abnormal behavior. Increasing robustness by selective breeding, therefore, improves welfare by adapting animals to the production environment. This does, however, not take away the need for improvement of housing conditions.

Christiansen and Sandøe (2000) mentioned that breeding for animals that are better suited for intensive farming instead of adapting the farming system may be considered violations of animal integrity. This, however, is only the case in those situations where adapting the animal involves diminishing its ability to live a good life or by depriving the animals of natural abilities, such as being able to see. However, improving the ability to cope with stress and improving the ability to recover by using robustness as a breeding goal does not deprive natural abilities, and is, therefore, not a violation of animal integrity. Of course, we have to be aware that when selecting for robust laying hens it is unknown if problems negatively correlated with the genetic make-up underlying robustness will occur.

CONCLUSION

The aim of this paper was to develop the concept of robustness as a breeding goal. Improving robustness by selective breeding will increase (or restore) the animals' ability to interact successfully with the environment and thereby to make the animal better able to adapt to an appropriate husbandry system. This, in turn, is likely to improve both welfare and productivity, although this also depends on management and housing conditions.

The implementation and application of robustness as a breeding goal is desirable. We are convinced that this application will result in animals with improved health and welfare without affecting the integrity. Therefore, improving robustness by introducing this concept as a breeding goal is ethically acceptable.

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CHAPTER 3

Natural humoral immune competence and survival in layers

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ABSTRACT

The relation between survival and levels of humoral components of innate (and specific) immune competence of laying hens was investigated in a population of 1,063 laying hens from twelve purebred layer lines. Natural immune competence of the chickens was studied by measuring levels of natural antibodies (NAb) binding to keyhole limpet haemocyanin (KLH) or lipopolysaccharide (LPS), respectively, and haemolytic (classical and alternative) complement activity at 20, 40, and 65 wk of age. In addition, levels of antibodies binding a Newcastle disease (NCD) vaccine strain as a measure of specific immunity were investigated at 20 wk of age. A distinction could be made between lines showing high or low immune competence with respect to NAb, complement activity, and specific antibodies. Within lines, significant correlations were found for each of the innate parameters among the three ages. The innate and specific parameters were, however, not correlated with each other. Based on the limited data set, it was not possible to draw conclusions on line differences for innate or specific immune competence in relation to survival. However, regardless of line, low levels of NAb binding to KLH or high levels of NAb binding to LPS were detected in chickens that did not survive the laying period. The major difference between the responses of NAb binding to KLH or LPS was that the chickens probably did not encounter KLH, which suggests a reflection of the capacity to respond, whereas the chickens most probably did encounter LPS, which suggests a reflection of the active status of the innate humoral immune system. In conclusion, we propose that levels (KLH) and activation (LPS) of components of natural antibodies are indicative for the probability that chickens survive a laying period.

Key words: natural immunity, natural antibody, haemolytic complement activity, specific antibody, survival rate

Abbreviation key: APW = alternative complement pathway; CPW = classical complement pathway; KLH = keyhole limpet haemocyanin; LPS = lipopolysaccharide; NAb = natural antibody; NCD = Newcastle disease; SRBC = sheep red blood cells

INTRODUCTION

Innate (natural) immunity is the most universal, rapid acting, and probably, the most important part of immunity. Many organisms survive through innate (-like) immune systems alone; in vertebrates, however, an additional acquired immunity evolved (Beutler, 2004). In the absence of an innate immune system, however, acquired immunity would offer weak or delayed protection, because skewing and maintenance of the acquired immune response and even the most pronounced characteristic of specific immunity, memory, rests to an important degree on innate mechanisms (Bernasconi et al., 2003). Finally, the innate immune system usually acts effectively without previous exposure to a pathogen and confers broad protection against a variety of pathogens (Kimbrell and Beutler, 2001).

Among the first line of innate immune defense two (interrelated) humoral components can be distinguished: natural antibodies (NAb) and the complement system. The NAb are defined as antigen-binding antibodies present in non-immunized individuals, which have a broad specificity repertoire and usually a low binding affinity, and which can be directed to exogenous as well endogenous antigens (Boes, 2000; Ochsenbein et al., 1999). The NAb are potentially important biological agents, prevalent in the healthy immune repertoire (Bayry et al., 2005) and are proposed to participate in the maintenance of immune homeostasis by exposure to environmental stimulations (Coutinho et al., 1995). It has been shown that NAb enhance specificity on the humoral and cellular levels in chicken (Lammers et al., 2004). In addition, NAb might be prerequisite to modulate the T-helper 2 route of specific immunity by maturation of dendritic cells (Bayry et al., 2004,2005). The complement system is a complex enzymatic system consisting of more than 30 proteins. These proteins participate in a cascade to defend the host against invading pathogens. There are three pathways for activating the complement system: the classical (CPW), alternative (APW) and mannan-binding lectin pathway, all resulting in formation of a membrane attack complex (Parmentier et al., 2002; Walport, 2001a). Formation of an antibody-antigen complex is the principle way of activating the classical complement pathway (Walport, 2001b), whereas the alternative pathway is activated directly by foreign microbial particles (Parmentier et al., 2002), and the mannan-binding lectin pathway is activated by foreign microbial particles following their binding by recognition molecules such as mannan-binding lectin (Carroll and Prodeus, 1998; Laursen and Nielsen, 2000). The NAb (Ochsenbein et al., 1999; Ochsenbein and Zinkernagel, 2000; Stäger et al., 2003) and complement (Thorbecke et al., 1994) have been shown to perform important functions in

the subsequent activation of specific humoral and cellular immune responses in mammals. The presence and activities of NAb (Lammers et al., 2004; Parmentier et al., 2004b) and complement (Laursen and Nielsen, 2000; Parmentier et al., 2004a) in poultry were reported earlier.

Innate immunity might play an important role in enhancing survival of the host by providing early resistance against infection (Ochsenbein and Zinkernagel, 2000). Low levels of innate immunity, cellular as well as humoral, may be related with disease susceptibility and high levels with disease resistance (Parmentier et al., 2004a); however, a relation between innate immunity and survival has not been shown before.

An experiment was conducted to investigate a relation between survival and humoral components of innate (natural) as well as specific immune competence of laying hens during one laying period. Natural immune competence of the chickens was studied by measuring levels of NAb and haemolytic complement activity (CPW and APW). Antigens were chosen to estimate levels of NAb binding to an exo-antigen (keyhole limpet haemocyanin, KLH), which the chickens have probably not encountered before nor will encounter during life, or an environmental-antigen (lipopolysaccharide, LPS) derived from the intestinal gram-negative micro biota. Specific immune reactivity was studied by measuring antibody levels binding to Newcastle disease (NCD) virus vaccine strain to which chickens have been routinely vaccinated at an earlier age. In the present study we established differences between layer lines in levels of natural and specific humoral immune competence, but most importantly, our data suggests that, regardless of line, levels of NAb binding to KLH or LPS were related to the probability of surviving the laying period.

MATERIALS AND METHODS

Chickens, housing, and feed

A population of 1,063 laying hens was used to establish natural and specific antibody levels and haemolytic complement activity. Within this population twelve purebred layer lines (Hendrix Genetics, Boxmeer, the Netherlands) could be distinguished: six White Leghorn lines (W1, WA, WB, WC, WD, and WF) and six Rhode Island Red lines (B1, B2, B3, BA, BB, and BE).

Chickens arrived at the laying facility at 17 wk of age and were housed in battery cages with four chickens of the same line in each cage (44 cm height × 46 cm depth × 39 cm

width). All chickens were housed in the same facility to minimize variation in environmental influences.

At the start of the laying period (at 19 wk of age) until 42 wk of age, chickens were fed a standard commercial phase 1 diet (159 g/kg crude protein, 43 g/kg crude fibre, and 11.17 MJ ME/kg). From 42 wk until the end of the laying period (at 70 wk of age), chickens were fed a standard commercial phase 2 diet (152 g/kg crude protein, 47.0 g/kg crude fibre, and 11.01 MJ ME/kg). Chickens had free access to feed and water.

At 17 wk of age chickens were kept at 9L:15D light scheme. After one wk, the light period was increased with half an hour. Hereafter, the light period was increased with approximately 10 min/d. From 30 wk onwards chickens were kept at 16L:8D light scheme.

Chickens were not debeaked. Chickens received routine vaccinations to Marek's disease (d 1), NCD (wk 2, 6, 12, 15), infectious bronchitis (d 1, wk 2, 10, 12, 15), infectious bursal disease (wk 3, 15), fowl pox (wk 15), and avian encephalomyelitis (wk 15).

EXPERIMENTAL DESIGN

The observational period consisted of one laying period, from 20 until 70 wk of age. During this period a population of 2,504 chickens [range 180 to 244 chickens per line (respectively, 45 to 61 cages per line) at the start of lay] was monitored, and from chickens that died during the laying period the day of mortality was registered (cause of death was not determined).

From this population of 2,504 chickens, 1,063 chickens were used to establish natural and specific antibody levels and haemolytic complement activity. Blood samples from approximately 80 chickens per line [range 74 to 86 per line (respectively, 18.5 to 21.5 cages per line) at the start of the experiment] were taken from a wing vein at 20, 40, and 65 wk of age for measurement of immune parameters. At each sample moment the same chickens were used. Serum was collected and stored at -20ºC for further processing.

The NAb binding to KLH or LPS, and complement activity of the classical and alternative pathways, were determined at 20, 40, and 65 wk of age. Specific antibodies binding to NCD vaccine were determined at 20 wk of age.

IMMUNE PARAMETERS

NATURAL ANTIBODIES. The NAb binding to KLH or LPS were determined in individual samples by an indirect ELISA procedure as published earlier (Parmentier et al., 2004b). Flat-bottomed 96-well medium binding ELISA-plates were coated with 1 µg/ml of KLH (MP Biomedicals Inc., Aurora, Ohio, 44202), or 8 µg/ml of *Escherichia coli* LPS (serotype

O55:B5, Sigma Chemical Co., St. Louis, MO). After subsequent washing the plates were filled with 100 µl phosphate buffered saline + Tween (0.05%) + newborn calf serum (2%) per well. Serum samples were stepwise diluted (1:30, 1:90, 1:270, and 1:810), and the plates were incubated for one h at room temperature. Binding of the antibodies to KLH or LPS antigen was visualized using a 1:20,000 diluted rabbit anti-chicken IgG_{H+L} labelled with peroxidase (RACh/IgG_{H+L}/PO; Nordic, Tilburg, the Netherlands). After washing, substrate (tetramethylbenzidine and 0.05% H_2O_2) was added, and 10 min later, the reaction was stopped with 2.5 N H_2SO_4. Extinctions were measured with a Multiskan (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Levels (titers; i.e., equivalent to double amounts of antibodies in serum) were calculated based on $\log_2$ values of the dilutions that gave extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of a standard positive serum present on each plate.

Complement assay. Sera were diluted in appropriate buffers in flat-bottomed 96-well micro titer plates and incubated with sensitized (Haemolysin, Biomerieux, ref. no. 72202) SRBC to measure CPW, or bovine red blood cells to measure APW as published earlier (Demey et al., 1993; Parmentier et al., 2002). During 1.5 hour of incubation the plates were shaken every 30 min. After that the plates were read with a Multiskan at a wavelength of 690 nm. Readings were transformed by log-log equation (Von Krogh, 1916), and the haemolytic titer was expressed as the titer that lyses 50% of the red blood cells (CH50 U/ml).

Specific antibody response to newcastle disease vaccine. Sera collected at 20 wk of age (i.e., 5 wk after the last NCD vaccination) were tested for the levels of specific antibodies to the vaccine strain (NCD clone 30, Intervet International BV, Boxmeer, the Netherlands) using an indirect ELISA as described above. Plates were coated with 100 µl/well of a solution of 1,000 doses of NCD dissolved in 200 ml of carbonate buffer. Sera were diluted 1:40, 1:160, 1:640, and 1:2,560, respectively.

Survival

The population of 2,504 chickens (range 180 to 244 chickens per line at the start of lay) was monitored, including the chickens that were used for blood collection. From chickens that died during the laying period the day of mortality was registered (cause of death was not determined). In addition, for the same twelve lines mortality data had been registered in the laying period of one yr earlier. Mortality data during the earlier laying period were

based on a different population of chickens (between 144 and 488 chickens per line at the start of lay). The main difference between the earlier laying period and the laying period in the present study was that the chickens in the earlier laying period were debeaked, and in the laying period of the present study chickens were not debeaked.

STATISTICAL ANALYSIS

A one-way ANOVA was performed to investigate differences in levels of NAb binding to KLH or LPS, and in activity of the classical and alternative complement pathway in the twelve purebred layer lines at each sample time (20, 40, and 65 wk of age). Differences among the twelve lines for specific antibodies binding to NCD were only investigated at 20 wk of age. Mean differences among lines were tested with Bonferroni's test.

Differences in rank number of the lines for the various natural immune parameters were tested with the Wilcoxon signed-rank test for each parameter at 20, 40, and 65 wk of age. At 20 wk of age, differences in rank number were tested between the natural immune parameters and specific antibodies binding to NCD. At 40 and 65 wk of age, differences in rank number were tested between the natural immune parameters.

Correlation between levels of NAb binding to KLH or LPS and activity of the classical and alternative complement pathway at 20, 40, and 65 wk of age were analyzed for each of the twelve layer lines by Pearson product-moment correlation. Correlation between the natural immune parameters and specific antibodies binding to NCD were analyzed for each of the twelve layer lines at 20 wk of age.

To study the relation between survival [binary variable taking the values 0 (survived) or 1 (died)], and natural (levels of NAb and complement activity) or specific immune competence (level of antigens directed to NCD), univariable logistic regression analysis was applied first. Variables with $P < 0.25$ were included in a multivariable logistic regression model:

$$\text{logit}(\pi) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \varepsilon,$$

where logit $(\pi) = \ln(\pi/1-\pi)$ and π is the probability to die given a set of explanatory variables: $P(Y=1|X)$, x_1 = effect of line, x_2 = effect of natural immune parameter 1 (at 20 or 40 wk of age), and x_3 = effect of natural immune parameter 2 (at 20 or 40 wk of age). Outcomes will be presented as odds ratios, which indicate the relative change in risk to die dependent on the levels of natural immune parameters.

For the univariable as the multivariable logistic regression analysis, the laying period was divided in three parts (20 to 39 wk of age, 40 to 64 wk of age, and 65 to 70 wk of age)

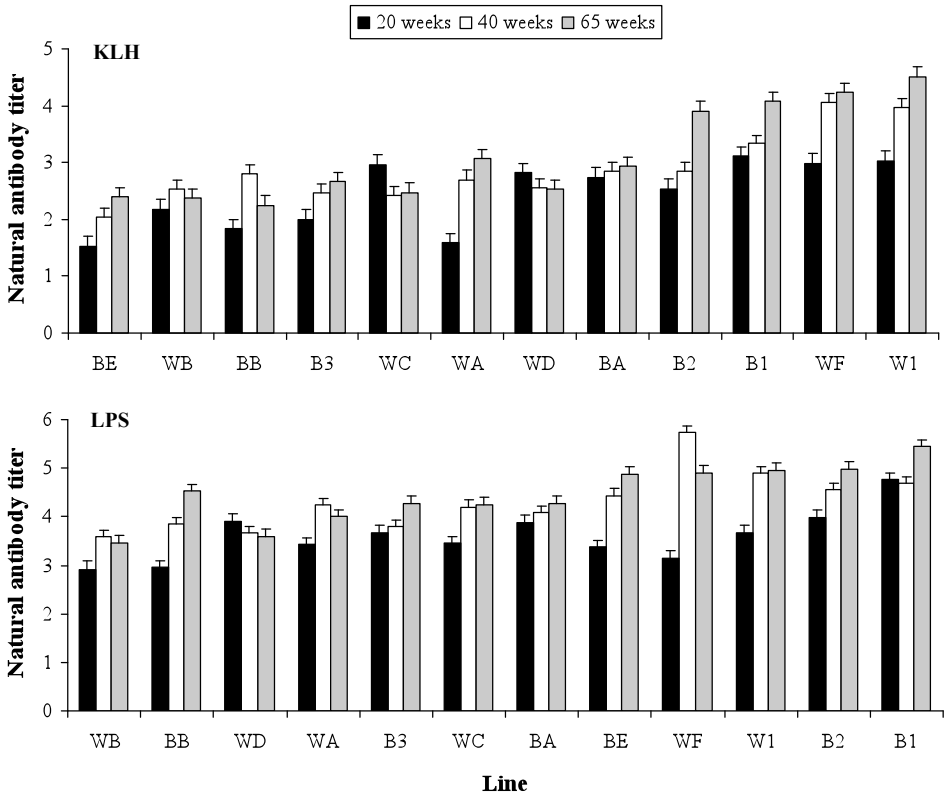

FIGURE 3.1. Levels of natural antibodies binding to keyhole limpet haemocyanin (KLH) or lipopolysaccharide (LPS) in twelve purebred layer lines: six White Leghorn lines (W1, WA, WB, WC, WD, and WF) and six Rhode Island Red lines (B1, B2, B3, BA, BB, and BE). Values are least square means (log_2 value + SE) of natural antibody titers determined in serum samples collected from approximately 80 chickens per line at 20, 40, and 65 wk of age (at each sample moment the same chickens were used). Lines are distributed according to total rank number per tested antigen (sum of rank numbers determined at 20, 40, and 65 wk of age).

depending on the time of blood sampling. Chickens that died between 20 and 39 wk of age were tested against surviving chickens for natural immune parameters measured in serum collected at 20 wk of age, and chickens that died between 40 and 64 wk of age were tested against surviving chickens for natural immune parameters measured in serum collected at 40 wk of age. Chickens that died after 65 wk of age were not tested because of the low mortality rate in the last weeks of lay. A logistic regression analysis to test the predictive

TABLE 3.1. Pearson correlation coefficients (r) (and P-values) between 20 and 40, 20 and 65, and 40 and 65 wk of age for keyhole limpet haemocyanin (KLH) or lipopolysaccharide (LPS) in twelve pure bred layer lines: six White Leghorn lines (W1, WA, WB, WC, WD, and WF) and six Rhode Island Red lines (B1, B2, B3, BA, BB, and BE)

	20 × 40		20 × 65		40 × 65	
Line	r	P-value	r	P-value	r	P-value
			KLH			
B1	0.54	<0.0001	0.47	<0.0001	0.65	<0.0001
B2	0.46	<0.0001	0.44	0.0002	0.49	<0.0001
B3	0.60	<0.0001	0.49	<0.0001	0.68	<0.0001
BA	0.49	<0.0001	0.46	<0.0001	0.82	<0.0001
BB	0.33	0.0044	0.25	0.0448	0.68	<0.0001
BE	0.27	0.0246	0.11	0.3808	0.66	<0.0001
W1	0.33	0.0047	0.20	0.1231	0.51	<0.0001
WA	0.43	0.0001	0.46	<0.0001	0.67	<0.0001
WB	0.30	0.0130	0.32	0.0114	0.53	<0.0001
WC	0.49	<0.0001	0.53	<0.0001	0.84	<0.0001
WD	0.33	0.0035	0.28	0.0168	0.74	<0.0001
WF	0.35	0.0030	0.28	0.0252	0.66	<0.0001
			LPS			
B1	0.23	0.0415	0.19	0.1037	0.59	<0.0001
B2	0.50	<0.0001	0.52	<0.0001	0.72	<0.0001
B3	0.47	<0.0001	0.48	<0.0001	0.79	<0.0001
BA	0.41	0.0003	0.57	<0.0001	0.46	<0.0001
BB	0.39	0.0005	0.27	0.0300	0.35	0.0049
BE	0.29	0.0151	0.35	0.0026	0.58	<0.0001
W1	0.41	0.0004	0.19	0.1462	0.56	<0.0001
WA	0.50	<0.0001	0.46	<0.0001	0.61	<0.0001
WB	0.59	<0.0001	0.40	0.0012	0.65	<0.0001
WC	0.21	0.0896	0.22	0.0929	0.62	<0.0001
WD	0.36	0.0013	0.50	<0.0001	0.62	<0.0001
WF	0.42	0.0003	0.35	0.0033	0.50	<0.0001

value of natural immunity at 20 wk of age at survival during the whole laying period was also performed.

Analysis of line differences, differences in rank number, and correlations were carried out using SAS (SAS Institute, 2004). Logistic regression analysis was carried out using Stata 8 (StataCorp, 2003). Effects were considered significant at $P < 0.05$.

RESULTS

Immune competence

Natural antibodies. Average levels of NAb binding to KLH or LPS in chickens at 20, 40, and 65 wk of age differed between lines (Figure 3.1). In ten of the twelve and eleven of the twelve lines, respectively, an increase in levels of NAb binding to KLH and LPS was found with increasing age (from 20 to 65 wk of age). Ranking of the lines for NAb binding to KLH or LPS were similar ($P > 0.05$) irrespective of aging. Chickens of lines B1, W1, and WF showed the highest levels of NAb for each antigen at each age (except line WF for NAb binding to LPS at 20 wk of age). In contrast to these high lines were lines BB and WB; chickens of these lines showed the lowest levels of NAb for each antigen at each age.

Within each line, significant correlations were found for levels of NAb binding to KLH or LPS between 20 and 40 wk of age and 40 and 65 wk of age. Correlations between NAb binding to KLH or LPS at 20 and 65 wk of age were significant in ten of the twelve lines and nine of the twelve lines, respectively. The age-related correlations for NAb binding to KLH or LPS are given in Table 3.1. There were no correlations of interest between NAb binding to KLH and LPS (data not shown).

Haemolytic complement activity. Average haemolytic complement activity of the classical and alternative complement pathways in chickens at 20, 40, and 65 wk of age differed between lines (Figure 3.2). In each line an increase in CPW and APW activity was found with increasing age (from 20 to 65 wk of age). This was mainly due to the strong increase of CPW and APW activity at 65 wk of age compared with CPW and APW activity at 20 and 40 wk of age. Ranking of the lines over the three ages seems less constant, although the ranking of lines for complement activity was not significantly different among the three ages and between complement activity and NAb levels. Again, line WF was among the highest ranked lines for CPW (except at 65 wk of age) and APW. Lines WB and WD were ranked among the lowest lines.

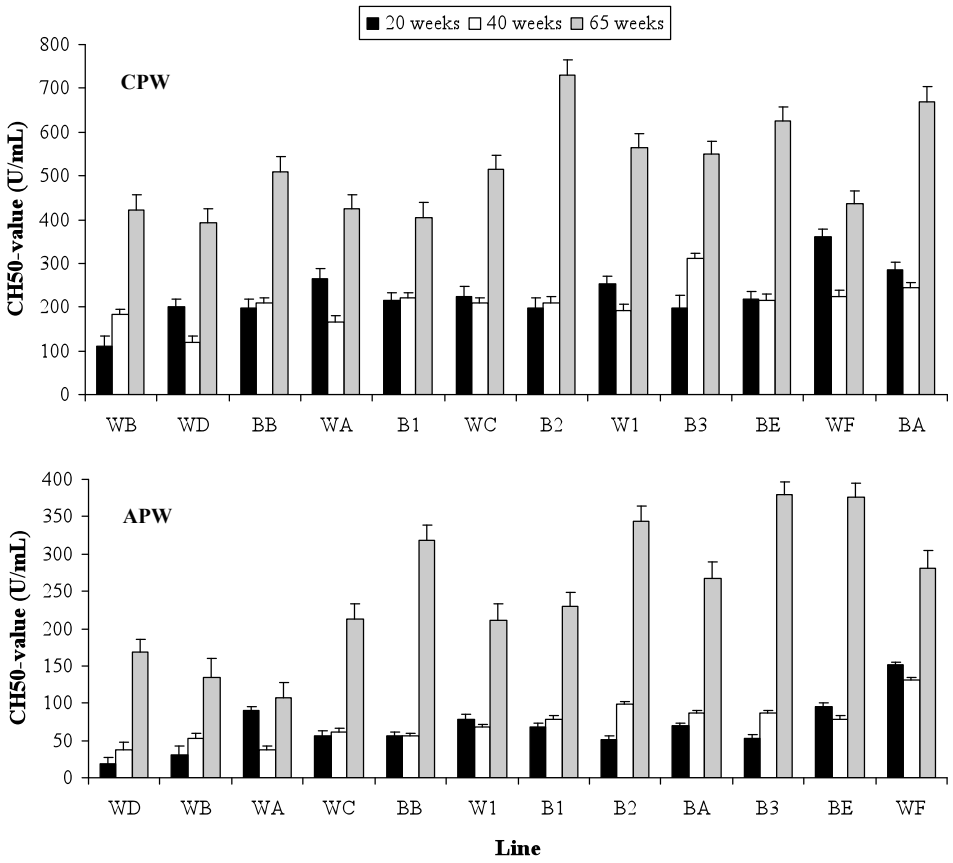

FIGURE 3.2. Haemolytic complement activity of the classical and alternative complement pathway (CPW and APW) in twelve pure bred layer lines: six White Leghorn lines (W1, WA, WB, WC, WD, and WF) and six Rhode Island Red lines (B1, B2, B3, BA, BB, and BE). Values are least square means [CH50-value (U/mL) + SE] of haemolytic complement activity determined in serum samples collected from approximately 80 chickens per line at 20, 40, and 65 wk of age (at each sample moment the same chickens were used). Lines are distributed according to total rank number per tested complement pathway (sum of rank numbers determined at 20, 40, and 65 wk of age).

Correlations for CPW or APW between 20 and 40 wk of age were only significant in three of the twelve and six of the twelve lines, respectively. Between 40 and 65 wk of age, significant correlations for CPW or APW were found in eight of the twelve and nine of the twelve lines, respectively. For CPW no significant correlations were between 20 and 65 wk

Table 3.2. Pearson correlation coefficients (r) (and P-values) between 20 and 40, 20 and 65, and 40 and 65 wk of age for the classical (CPW) and alternative (APW) complement pathway in twelve pure bred layer lines; six White Leghorn lines (W1, WA, WB, WC, WD, and WF) and six Rhode Island Red lines (B1, B2, B3, BA, BB, and BE)

	20 × 40		20 × 65		40 × 65	
Line	r	P-value	r	P-value	r	P-value
			CPW			
B1	0.24	0.0658	0.24	0.1266	0.53	<0.0001
B2	0.19	0.2848	-0.07	0.7317	0.16	0.2449
B3	-0.03	0.8930	0.07	0.7220	0.39	0.0006
BA	0.26	0.0441	0.27	0.0672	0.47	0.0001
BB	0.58	<0.0001	0.14	0.4280	0.34	0.0114
BE	0.01	0.9654	0.13	0.3492	0.13	0.3492
W1	0.21	0.1598	0.22	0.1634	0.53	<0.0001
WA	0.20	0.2513	0.12	0.5214	0.33	0.0149
WB	0.24	0.1681	-0.11	0.5623	0.11	0.4050
WC	0.38	0.0353	0.13	0.5339	0.06	0.6487
WD	0.03	0.8389	0.04	0.8112	0.38	0.0031
WF	0.26	0.0575	0.26	0.0653	0.33	0.0079
			APW			
B1	0.18	0.3574	0.48	0.0160	0.50	0.0001
B2	0.32	0.0953	0.21	0.3639	0.31	0.0488
B3	0.45	0.0035	0.45	0.0290	0.42	0.0029
BA	0.35	0.0215	0.14	0.5161	0.53	0.0008
BB	0.51	0.0057	0.27	0.2330	0.36	0.0410
BE	0.31	0.0318	0.25	0.0793	0.15	0.2608
W1	0.27	0.1614	0.56	0.0083	0.38	0.0260
WA	0.58	0.0178	0.28	0.1585	0.47	0.0276
WB	-	-	-0.18	0.7751	-0.23	0.4974
WC	0.27	0.3027	0.49	0.0292	0.69	<0.0001
WD	-	-	0.35	0.3282	0.46	0.2072
WF	0.43	0.0010	0.29	0.0500	0.44	0.0012

of age, whereas for APW between 20 and 65 wk of age a significant correlation was found in four of the twelve lines. The age-related correlations for CPW and APW are given in Table 3.2. Besides correlations for CPW or APW, correlations were also significant between CPW and APW at 40 and 65 wk of age in eleven of the twelve and nine of the twelve lines, respectively (data not shown). There were no correlations of interest between CPW and APW at 20 wk of age. Furthermore, there were no correlations between complement activity (CPW or APW) and levels of NAb (binding to KLH or LPS; data not shown).

SPECIFIC ANTIBODY RESPONSE TO NEWCASTLE DISEASE VACCINE. Levels of specific antibodies binding to NCD vaccine were only analyzed at 20 wk of age because the chickens were vaccinated for the last time with NCD 5 wk earlier. The highest and lowest antibody level was found in, respectively, lines B1 (average level of 7.46) and WB (average level of 4.61; Figure 3.3). Although correlations were not significant between specific antibodies binding to NCD and each of the natural immune parameters at 20 wk of age, ranking of the lines for average antibody levels to NCD was similar to ranking of the lines for NAb.

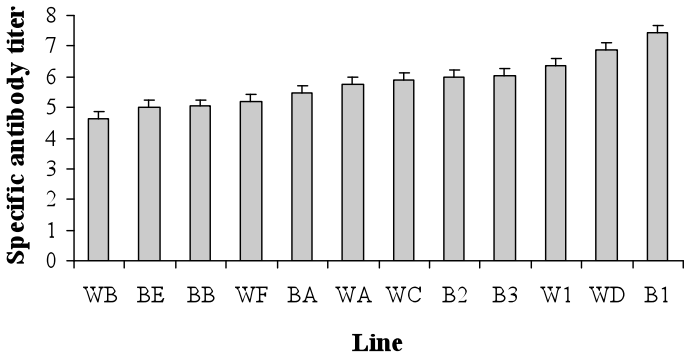

FIGURE 3.3. Specific antibodies binding to a vaccine for Newcastle disease (NCD) in twelve pure bred layer lines; six White Leghorn lines (W1, WA, WB, WC, WD, and WF) and six Rhode Island Red lines (B1, B2, B3, BA, BB, and BE). Values are least square means (log_2 value + SE) of specific antibody titers determined in serum samples collected from approximately 80 chickens per line at 20 wk of age. Lines are distributed according to rank number for specific antibody titer to NCD.

Survival

Survival rate of the twelve layer lines in this experiment was between 81.5% (line WC) and 95.6% (line B3; Table 3.3) with an overall survival rate of 88.7%. In the earlier (trimmed beaks) and current (intact beaks) laying periods, the lines had a similar rank number with respect to survival, except line B3 that showed a large increase in survival rate, 88.9% and 95.6% for trimmed and intact beaks, respectively, resulting in a large difference in rank number for survival rate. Lines WA and WF had a high survival rate in both laying periods, and therefore overall a high rank number, whereas lines WC and WB had a low survival rate in both periods and therefore a low rank number.

Table 3.3. Number of animals at the start of the laying period (n), survival rate (%) (rank number) and survival days (± SD) of twelve purebred layer lines [six White Leghorn lines (W1, WA, WB, WC, WD, and WF) and six Rhode Island Red lines (B1, B2, B3, BA, BB, and BE)] as found in an earlier laying period done by Hendrix Genetics in beak-trimmed chickens and within the laying period of the present study (Experiment) in chickens with intact beaks

	Hendrix Genetics				Experiment			
Line	n	Survival		Survival days	n	Survival		Survival days
B1	235	89.8%	(6)	477	200	86.5%	(8)	345 ± 58
B2	340	93.8%	(2)	486	200	92.0%	(4)	351 ± 57
B3	144	88.9%	(8)	486	180	95.6%	(1)	360 ± 34
BA	488	90.4%	(5)	484	200	91.0%	(6)	349 ± 58
BB	266	87.6%	(10)	478	244	87.3%	(7)	348 ± 57
BE	385	88.6%	(9)	483	230	82.2%	(11)	338 ± 75
W1	249	89.6%	(7)	484	197	86.3%	(9)	347 ± 57
WA	250	97.6%	(1)	492	210	94.8%	(2)	357 ± 43
WB	340	87.1%	(11)	479	204	85.8%	(10)	343 ± 68
WC	378	87.0%	(12)	474	233	81.5%	(12)	339 ± 72
WD	279	92.1%	(4)	487	206	92.3%	(5)	352 ± 49
WF	212	92.4%	(3)	483	200	93.0%	(3)	351 ± 58

Natural immune competence indicative for probability to survive

To investigate the relation between survival and immune competence a logistic regression analysis was performed. Based on the limited data set, it was not possible to draw conclusions on line differences for innate or specific immune competence in relation to survival. Therefore, the logistic regression analysis was performed over lines, where the

TABLE 3.4. Outcome of the logistic regression model expressed as odds ratio and P-value of natural antibodies binding to keyhole limpet haemocyanin (KLH) or lipopolysaccharide (LPS) measured at 20 and 40 wk of age in surviving and non-surviving chickens of all twelve purebred layer lines during, respectively, the first period of lay (20 to 39 wk of age; natural humoral immune parameters included as continuous) and the second period of lay (40 to 64 wk of age; natural humoral immune parameters included as categorical)

	20 weeks		40 weeks		
Antigen	Odds ratio	P-value	Category	Odds ratio	P-value
KLH	0.80	0.008	titer < 2[1]		
			titer 2 to 3	0.33	<0.0001
			titer 3 to 4	0.66	0.256
			titer 4 to 5	0.25	0.001
			titer 5 to 6	0.61	0.159
			titer > 6	0.26	0.035
LPS	1.42	<0.0001	titer < 2[1]		
			titer 2 to 3	4.35	0.060
			titer 3 to 4	2.03	0.359
			titer 4 to 5	4.73	0.039
			titer 5 to 6	3.60	0.092
			titer > 6	7.86	0.009

[1] Reference category.

statistical model was corrected for line. For the logistic regression analysis the laying period was divided in three parts based on the time of blood sampling. Survival rates for the period from 20 to 39 wk of age and 40 to 64 wk of age were 96.2% and 94.1%, respectively. Chickens that died after 65 wk of age were not tested because of the low mortality rate in the last weeks of lay.

The level of NAb binding to KLH or LPS at 20 wk of age was, regardless of line, significantly related to the probability to survive the first period of lay (between 20 and 39 wk of age). Chickens with a lower level of NAb binding to KLH or a higher level of LPS had a lower probability to survive this period (Figure 3.4). For NAb binding to KLH an odds ratio of 0.80 was found (Table 3.4), which means that if NAb binding to KLH increases with 1 unit (titer; i.e., equivalent to double the amount of serum NAb) the relative change in risk to die during the first period of lay increases 0.80-fold (i.e., decreases with 20%). For NAb binding to LPS an odds ratio of 1.42 was found, which means that if NAb

binding to LPS increases with 1 unit (titer) the relative change in risk to die during the first period of lay increases 1.42-fold (i.e., increases with 42%).

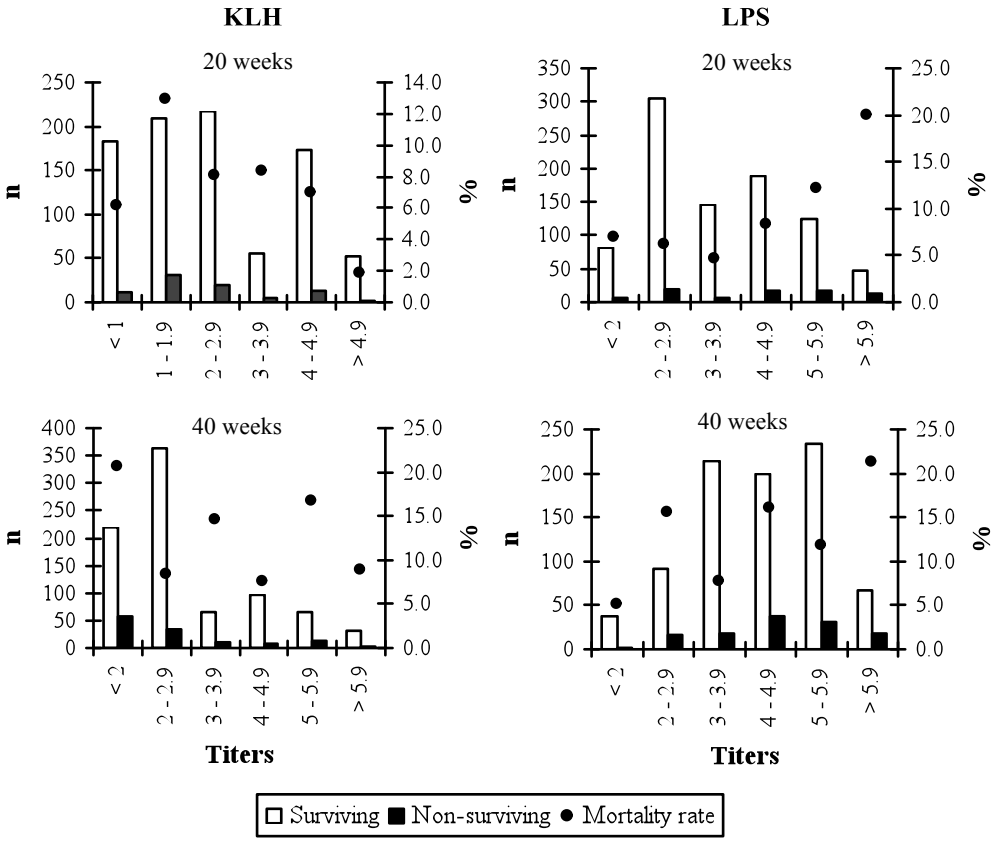

FIGURE 3.4. Mortality rate (%) and number (n) of surviving and non-surviving chickens during the first period (20 to 39 wk of age) and the second period of lay (40 to 64 wk of age) classified by levels of natural antibodies (log_2 value) binding to keyhole limpet haemocyanin (KLH) or lipopolysaccharide (LPS).

The level of NAb binding to KLH or LPS at 40 wk of age was, regardless of line, significantly related to the probability to survive the second period of lay (between 40 and 64 wk of age), but this was not a linear effect (Figure 3.4). Therefore, NAb binding to KLH and LPS was categorized into 6 classes (titer < 2, titer 2 to 3, titer 3 to 4, titer 4 to 5, titer 5 to 6, and titer > 6; Table 3.4). For NAb binding to KLH all classes had an odds ratio lower than 1 compared with the reference category (titer < 2), which means that hens with low

levels of NAb binding to KLH have a higher chance to die between 40 and 64 wk of age. For NAb binding to LPS all classes had an odds ratio higher than 1 compared with the reference category (titer < 2), which means that hens with high levels of NAb binding to LPS have a higher chance to die between 40 and 64 wk of age.

Levels of NAb binding to KLH or LPS at 20 wk of age were not significantly related to the probability to survive the whole laying period. Furthermore, neither activity of the classical and alternative complement pathway at 20 and 40 wk of age nor levels of specific antibodies binding to NCD vaccine at 20 wk of age were significantly related to the probability to survive (data not shown).

DISCUSSION

To study a relation between levels of innate (natural) humoral immune competence and survival in laying hens, levels of NAb and complement activity were investigated at three ages during one laying period. Antigens were chosen to estimate levels of NAb binding to an exo-antigen (KLH), which the chickens most probably have not encountered before nor will encounter during life, or an environmental-antigen (LPS) derived from the intestinal micro biota. It is likely that the chickens encountered LPS, but we have chosen to use the word natural for LPS-binding antibodies according to the definition that there is no intentional nor controllable challenge with the antigen leading to the formation of antibodies. Furthermore, because all chickens were housed in the same facility it was assumed that they were exposed to highly similar levels of environmental airborne or manure-derived LPS and that differences in levels of NAb binding to LPS could be attributed to line differences.

Most chickens, regardless of line, showed an increase in levels of NAb binding to KLH or LPS with aging. Higher levels of NAb with increasing age were reported before (Parmentier et al., 2004b), which correspond with the idea that exogenous stimuli enhance the formation of NAb (Prokešová et al., 1997) or maintain non-antigen-specific memory toward a variety of antigens that the individual has not encountered before and will be recognized by non-specific innate pathogen recognition receptors such as Toll-like receptors present on B-cells (Bernasconi et al., 2003). In this respect, levels of NAb binding to LPS may reflect environmental stimulation by ubiquitous bacteria present in micro biota or the air.

Complement activity (CPW and APW) was studied at three ages during the laying period. Demey et al. (1993) demonstrated that complement activity of chicken sera may decline due to storage time and storage temperature. In this experiment all serum samples were

stored at -20ºC, and storage time was comparable for all samples, enabling us to measure line differences. Line differences were significant for CPW and APW at 20, 40, and 65 wk of age. Furthermore, the lower complement activity as observed in younger birds suggested an effect of age. Little is known about the effect of age on complement activity, but an increase of total complement activity was found for human (Nagaki et al., 1980) and ovine (Oswald et al., 1990).

The NAb binding to KLH or LPS and complement activity (CPW and APW) revealed a similar ranking depending on lines. A distinction could be made between lines showing high or low levels of NAb and complement activity. However, no significant correlations were found between the two types of NAb (directed to KLH or LPS), nor between NAb on the one hand and complement activity on the other hand. A relation between NAb and complement was expected because formation of an antibody-antigen complex is the principle way of activating the classical complement pathway (Walport, 2001b). Within lines, however, there were positive correlations for these parameters between the different ages. These results suggest that 1) NAb binding to KLH and LPS represent different functional B cell activities, 2) high levels of NAb can be found in chicken lines with high complement activity and low levels of NAb can be found in chickens with low complement activity, which fit with earlier observations of Parmentier et al. (2002,2004b), suggesting different mechanisms underlying the formation of NAb or complement, and 3) most importantly, chickens regardless of age act immunologically consistent over time (i.e., our findings are not due to an artifact caused by age), and therefore they may represent an immune phenotype and possibly genotype.

Innate and specific immunity are linked by both complement and NAb (Carroll and Prodeus, 1998; Ochsenbein and Zinkernagel, 2000). The NAb and complement were shown to perform important functions in the subsequent activation of specific humoral and cellular immune responses after vaccination (Lammers et al., 2004; Stäger et al., 2003). In the present study, chickens were not challenged with antigen, but specific antibody responses to NCD vaccination could be measured. Lines that showed high levels of NAb binding to KLH and LPS were also high responders for specific antibodies to NCD. Although there were no significant correlations between the natural immune parameters (NAb binding to KLH or LPS, and CPW and APW) and levels of specific antibodies to NCD, these data suggest (based on ranking) a genetic or functional linkage between natural immune competence and specific immune competence. It is worth mentioning, that NAb may be prerequisite to modulate the T-helper 2 route of specific immunity by maturation of dendritic cells (Bayry et al., 2004,2005).

Although the cause of death is unknown in this experiment, it is certain that most of the chickens died because of non-specific causes (no disease-related mortality or cannibalism). For non-specific mortality, therefore, non-specific (innate) immunity might play an important role in enhancing survival of the host by providing early resistance against infection (Ochsenbein and Zinkernagel, 2000). Low levels of innate immunity, cellular as well as humoral, may be related with disease susceptibility and high levels with disease resistance (Parmentier et al., 2004a); however, a relation between levels of or activation of natural immunity and survival has not been shown before. In knock-out mice (BALB/c), however, absence of NAb caused a delayed specific (T-dependent) response resulting in increased mortality (Baumgarth et al., 1999), which indicates the important role of the natural immune system for survival.

The major difference between the responses of NAb binding to the exo-antigens, KLH or LPS, is that chickens did not, and probably will not encounter KLH, thus reflecting a capacity to respond, whereas the chickens most probably did encounter LPS, thus the latter reflects an active status of the innate immune system. The present data suggest, regardless of line, a relation between levels (KLH) and activation (LPS) of humoral components of innate immunity and survival. Low levels of NAb binding to KLH were detected in chickens that did not survive, which suggests the (lack of) capacity to maintain NAb to KLH. Because KLH is a classical antigen for NAb and because it seems that (too) low levels of NAb binding to KLH are not in favor of survival, it is supposed that NAb binding to KLH reflects the capacity to mount an appropriate level of natural immune defense. Conversely, NAb binding to LPS reflect the immune reactivity of the chicken. The LPS is a gram-negative bacterial membrane molecule, but when released by damaged or dead bacteria, LPS could act as a danger signal crucial for stimulation of the innate immune response (Matzinger, 2002; Reid et al., 1997). Therefore it is supposed that chickens that did not survive had physiological or immunological problems at the time of blood sampling, and this is reflected by the levels of NAb binding to LPS.

In conclusion, a distinction could be made between lines showing high or low immune competence, with respect to NAb, complement activity, and specific antibodies. Within lines significant correlations were found for each of the innate and specific parameters among the three ages. The innate and specific parameters were, however, not correlated with one another. Based on the limited data set, it was not possible to draw conclusions on line differences for innate or specific immune competence in relation to survival. However, we found a relation, regardless of line, between natural immune competence (in the form of NAb binding to KLH) and activation of natural immunity (in the form of NAb binding to

LPS) and the probability to survive. The NAb reflecting immune competence or immune activation could thus be indicative factors for the chickens' abilities to survive a laying period within a genetic setting. To our knowledge this is the first study indicating a relation between innate immunity and survival. In further research, relations between NAb levels, disease susceptibility, and environmental influences (such as exposure to LPS) should be further unraveled.

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CHAPTER 4

Effect of single or combined climatic and hygienic stress on natural and specific humoral immune competence in four layer lines

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ABSTRACT

Effects of long-term climatic stress (heat exposure), short-term hygienic stress [lipopolysaccharide (LPS)], or a combination of both challenges on the immune competence of 4 layer lines was investigated. The lines were earlier characterized for natural humoral immune competence and survival rate. Eighty hens per line were randomly divided over 2 identical climate chambers and exposed to a constant high temperature (32ºC) or a control temperature (21ºC) for 23 d. Half of the hens housed in each chamber were i.v. injected with LPS at d 1 after the start of the heat stress period. Within each of the treatment groups, half of the hens were s.c. immunized with human serum albumin (HuSA) at d 2 after the start of the heat stress period to measure specific antibody (Ab) titers to HuSA. The effect of heat, LPS, or a combined challenge on specific Ab titers to HuSA, natural Ab titers to keyhole limpet haemocyanin or HuSA (in hens that were not immunized with HuSA), and activity of the classical and alternative complement pathways were investigated. Heat stress enhanced specific and natural immune responses. Administration of LPS enhanced natural immune responses but decreased specific immune responses. The lack of interaction between heat stress and LPS administration, except for natural Ab titers to HuSA, suggest that these were two independent stressors. The lines had a similar response pattern but differed in the response level. Neither natural humoral immune competence nor survival rate, for which the lines had been characterized, was indicative of the specific and natural immune response to different stressors. Lipopolysaccharide and heat stress initiated sequential responses over time, with an earlier effect of short-term LPS exposure (within the first and second week) and a later effect of long-term heat exposure (within the second and third week). These data suggest that LPS and heat stress affect the natural and specific immune competence of laying hens.

Key words: heat stress, haemolytic complement activity, lipopolysaccharide, natural antibody, specific antibody

Abbreviation key: Ab = antibody; APW = alternative complement pathway; CPW = classical complement pathway; HuSA = human serum albumin; KLH = keyhole limpet haemocyanin; LPS = lipopolysaccharide

INTRODUCTION

Genetic background, environmental conditions, and their interactions influence the immunological responsiveness of animals (Gross and Siegel, 1988). Coping with environmental changes is often associated with some degree of immune suppression or immune enhancement, depending on the type, duration, and intensity of the stressor. It has been suggested that chronic stress is associated with immune-suppressive effects, whereas acute stress is associated with immune-enhancing effects (Dhabhar and Viswanathan, 2005).

Several studies have been conducted evaluating the effects of high temperature (heat stress) on the immune responses of chickens. The heterophil to lymphocyte ratio has been used as a sensitive indicator of stress, including heat stress, among chicken populations (Gross and Siegel, 1983; Mashaly et al., 2004). Heat exposure resulted in an increased heterophil to lymphocyte ratio (Mashaly et al., 2004; McFarlane and Curtis, 1989), which indicates a relationship between heat stress and non-specific immune reactive cells (heterophil cells) (Mahmoud and Yaseen, 2005). To our knowledge, this relation is not found for other parts of the non-specific immune system, for instance, natural antibodies (Ab) and complement activity. We assume that these humoral parts of the non-specific immune system will also be affected by heat stress. Hangalapura et al. (2003,2004b) observed that the immune system of chickens exposed to cold stress responded immediately with enhanced levels of natural Ab. Furthermore, heat stress affects the specific immune response. Thaxton et al. (1968) were the first to demonstrate that a high environmental temperature (41 to 45ºC) affected the specific humoral immune response in young chickens. The decrease of specific Ab by exposure to heat stress was later also reported in broilers (Zulkifli et al., 2000) and laying hens (Mashaly et al., 2004). The results of heat stress on specific immune responses, however, were not consistent. Heller et al. (1979) found significantly increased Ab titers following heat exposure, whereas Donker et al. (1990) found that heat exposure did not affect Ab titers. Differences in immune responsiveness to heat stress may depend on the duration and intensity of the heat exposure (Kelley, 1985), breed of chicken (Regnier et al., 1980), or presence of other stressors experienced at the same time.

Inhalation of environmental gram-negative bacteria (in particular their endotoxin) is a major poultry health problem (Zucker et al., 2000), because these bacteria are ubiquitous in the environment of poultry (Chapman et al., 2005). Endotoxins may affect the type and magnitude of the immune responses in poultry, which is of major importance for

vaccination and health strategies (Maldonado et al., 2005). Administration of endotoxin (lipopolysaccharide, LPS) to chickens involves immune stimulation, such as release of interleukin-1 (Klasing and Peng, 1987) and tumor necrosis factor (Gehad et al., 2002), expression of Toll-like receptors 2 and 4 (Eicher and Cheng, 2003), and modulation of Ab responses (Gross and Siegel, 1975; Maldonado et al., 2005; Parmentier et al., 1998,2004c). These studies with LPS have been performed mostly on young poultry (6 d through 6 wk) in broilers or laying pullets. To our knowledge, the effect of microbial challenges, mimicked by LPS, on the innate and specific humoral immunity of adult laying hens has not been studied. Furthermore, the effects and interactions of a combined heat stress and microbial challenge on humoral immunity, in the form of natural Ab and complement activity, have never been studied in poultry, to our knowledge. It is, however, conceivable that interactions between heat exposure and microbial challenges commonly occur in modern poultry farming.

In a previous study (Star et al., 2007a), differences in natural humoral immunity were investigated in 12 purebred layer lines. For the present experiment, 4 of the 12 lines were selected based on high or low natural immune competence and a high or low survival rate. These lines were exposed to the following environmental stressors: heat (climatic stress), LPS (hygienic stress), or a combined exposure to heat and LPS. We hypothesized that chickens are able to cope with single environmental stressors, but that problems in coping ability occur when chickens are exposed to combined environmental stressors. Because the lines were selected for natural humoral immune competence and survival rate, the present paper will focus on effects of single exposure to high temperature or LPS administration, or combined exposure to both stressors on natural and specific humoral immune competence in these 4 layer lines. Natural humoral immune competence was studied in the form of natural Ab binding to keyhole limpet haemocyanin (KLH) or human serum albumin (HuSA), and activity of the classical (CPW) and alternative complement pathway (APW). Immunization with HuSA was done to study specific humoral immune competence.

MATERIALS AND METHODS

Chickens, housing, and feed

Four purebred layer lines (Hendrix Genetics, Boxmeer, the Netherlands) were used: 3 White Leghorn lines (WA, WB, and WF), and 1 Rhode Island Red line (B1). These lines were characterized for low or high survival rate and low or high natural humoral immune competence as determined in a previous study (Star et al., 2007a). Line B1 was

characterized by a low survival rate and high natural humoral immune competence, line WA was characterized by a high survival rate and low natural humoral immune competence, line WB was characterized by a low survival rate and low natural humoral immune competence, and line WF was characterized by a high survival rate and high natural humoral immune competence.

At 22 wk of age, 80 hens per line (320 in total) were transported from a housing facility at Hendrix Genetics to 2 identical climate respiration chambers at Wageningen University. In each climate chamber, 40 hens per line were individually housed in battery cages (45 cm height × 40 cm depth × 24 cm width). The lines were randomly divided over the cages. Hens were fed a standard commercial phase 1 diet (15.9% crude protein, 3.9% crude fiber, and 11.8 MJ of ME/kg). At 22 wk of age, hens were kept under a 13L:11D light scheme. In the following 2 wk, the light period was increased by 1 h. At the start of the experimental period (at 24 wk of age), hens were kept under a 15L:9D light scheme until the end of the experimental period (27 wk of age). Hens received routine vaccinations to Marek's disease (d 1), Newcastle disease (wk 2, 6, 12, 15), infectious bronchitis (d 1, wk 2, 10, 12, 15), infectious bursal disease (wk 3, 15), fowl pox (wk 15), and avian encephalomyelitis (wk 15). Beak trimming was not performed.

EXPERIMENTAL DESIGN

After an adaptation period of 12 d (temperature maintained at 21ºC), hens in the first climate chamber were exposed to acute heat stress. Within approximately 1 h, the temperature in this chamber was increased from 21 to 32ºC, and was maintained at 32ºC for the following 23 d. In the second chamber, the (control) temperature was maintained at 21ºC. At d 1 after the start of the heat stress period, half of the hens in the heat treatment and half of the control hens were i.v. injected with 1 mg/kg of BW of *Escherichia coli* LPS (serotype O55:B5, Sigma Chemical Co., St. Louis, MO). The remaining hens received a placebo treatment of PBS. At d 2 after the start of the heat stress period, half of the hens from each experimental group (4 treatment groups × 4 lines × 10 hens) were s.c. immunized with HuSA (Sigma Chemical Co.) to measure the specific Ab response of the hens. Hens that were not immunized with HuSA received a PBS placebo treatment. An overview of the experimental design is given in Table 4.1.

Table 4.1. Experimental design[1]

Item	Treatment group	Temperature (ºC)	HuSA[2]	Birds (n)
21ºC + PBS	Control natural antibody titer (NAb)	21	-	40
	Control specific antibody titer (SpAb)	21	+	40
21ºC + LPS	Effect LPS on NAb	21	-	40
	Effect LPS on SpAb	21	+	40
32ºC + PBS	Effect heat on NAb	32	-	40
	Effect heat on SpAb	32	+	40
32ºC + LPS	Effect heat and LPS on NAb	32	-	40
	Effect heat and LPS on SpAb	32	+	40

[1] There were 4 treatment groups, which were exposed to a temperature of 21 or 32ºC for 23 d, and were i.v. injected with *Escherichia coli* lipopolysaccharide (LPS) or PBS at d 1 after the start of the heat stress period. Within these 4 treatment groups half of the hens were s.c. immunized with human serum albumin (HuSA; control with PBS) at d 2 after the start of the heat stress period. Within each treatment group, 4 genetically different purebred layer lines, characterized by natural humoral immune competence and survival rate, were equally represented. Line B1 was characterized by a low survival rate and high natural humoral immune competence, line WA was characterized by a high survival rate and low natural humoral immune competence, line WB was characterized by a low survival rate and low natural humoral immune competence, and line WF was characterized by a high survival rate and high natural humoral immune competence.

[2] + = hens immunized with HuSA; - = hens not immunized with HuSA.

Immune parameters

Blood samples were collected from the wing vein of all 320 individual hens at d 5 prior to the start of heat stress and at d 2, 5, 8, 15, and 22 after the start of heat stress. After sampling, blood was centrifuged and serum was stored at -20ºC until further processing.

Humoral immune response to HuSA and KLH. Antibody titers to HuSA and KLH were determined in individual samples by an indirect ELISA procedure at d -5, 2, 5, 8, 15, and 22 after the start of the heat stress period. Flat-bottomed 96-well medium-binding ELISA plates were coated with 4 μg/mL of HuSA or 1 μg/mL of KLH (MP Biomedicals Inc., Aurora, OH). After subsequent washing the plates were incubated with serum (diluted 1:60, 1:360, 1:2,160, and 1:12,960 for HuSA, and diluted 1:30, 1:90, 1:270, and 1:810 for KLH). Binding of the Ab to HuSA and KLH antigen was visualized by using a 1:20,000 diluted rabbit anti-chicken IgG_{H+L} labeled with peroxidase (RACh/IgG_{H+L}/PO; Nordic, Tilburg, the Netherlands). After washing, substrate (tetramethylbenzidine and 0.05% H_2O_2)

was added, and 10 min later, the reaction was stopped with 2.5 *N* H_2SO_4. Extinctions were measured in a microplate reader (Multiskan, Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Levels (titers) were expressed as log2 values of the dilutions that gave extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of a standard positive serum present on each flat-bottomed ELISA-plate.

Haemolytic complement assay. Classical complement pathway and APW were determined in individual samples collected at d -5, 2, 5, 8, 15, and 22 after the start of the heat stress period. The haemolytic complement assay was performed according to the method described by Demey et al. (1993). Briefly, appropriate buffers were prepared. The buffer solution for CPW was prepared by adding $MgCl_2$ (1 mmol/L) and $CaCl_2$ (0.15 mmol/L) to veronal-buffered saline. The buffer solution for APW was prepared by adding $MgCl_2$ (5 mmol/L) and ethylene glycol tetraacetate (16 mmol/L) to veronal-buffered saline.

The assay was performed in flat-bottomed 96-well microtiter plates. Sera were diluted serially in the appropriate buffers and incubated with sensitized (ref. no. 72202, Haemolysin, bioMérieux, Marcy l'Etoile, France) sheep erythrocytes or bovine erythrocytes prepared by standard methods and used as a 1% cell suspension to measure CPW or APW, respectively. During 1.5 h of incubation, the plates were shaken every 30 min. The results (the amount of light scattering by erythrocytes upon lysis) were read in a microplate reader (Multiskan, Labsystems) at a wavelength of 690 nm. Readings were transformed by a log-log equation (Von Krogh, 1916), and the haemolytic titer was expressed as the titer that lyses 50% of the red blood cells (CH50 U/mL).

Statistical analysis

Differences in titers of natural and specific Ab binding to HuSA, natural Ab binding to KLH, and activity of CPW and APW were analyzed by a 4-way ANOVA for the effect of line, temperature, LPS administration, time, and their interactions by a repeated measurement procedure using a 'hen nested within line, temperature, and LPS administration' option. The effect of HuSA immunization was not included in the statistical model, because this immunization only affects the HuSA titer. Therefore, we choose to analyze hens immunized with HuSA separately from hens not immunized with HuSA. In this way, we were able to test whether temperature and LPS administration were of influence on the levels of specific Ab binding to HuSA in immunized hens, and whether these treatments were of influence on the levels of natural Ab binding to HuSA in non-immunized hens.

Mean differences among lines and treatments were tested with Bonferroni's test. The PROC MIXED of SAS was used for statistical analysis (SAS Institute, 2004). Effects were considered significant at $P < 0.05$.

RESULTS

Within 1 d after LPS administration, 3 hens died (1 of line B1 and 2 of line WA). These hens were exposed to the combined challenge of LPS and heat. All other hens survived the experimental period.

Ab responses to HuSA and KLH

Results of the effects of single or combined heat exposure and LPS administration on specific and natural Ab binding to HuSA, and on natural Ab binding to KLH in the 4 layer lines are shown in Table 4.2.

Specific Ab response to HuSA. Levels of specific Ab binding to HuSA were increased at d 8 and 15 after the start of the heat stress period (d 6 and 13 after HuSA immunization), and were still increased at d 22 after the start of the heat stress period, although the levels were lower than at d 8 and 15.

The total specific Ab response to HuSA was affected by LPS administration ($P < 0.0001$); at d 8, 15, and 22 after the start of the heat stress period, LPS-treated hens had a lower level of specific Ab to HuSA than PBS treated hens (LPS × time interaction; $P < 0.0001$).

The total specific Ab response to HuSA was affected by a line × heat × time interaction ($P < 0.0001$). Heat exposure enhanced the specific Ab response to HuSA at d 15 and 22 after the start of heat stress, except for line WB, in which heat-stressed hens had a lower specific Ab response to HuSA than hens from line WB exposed to the control temperature (Table 4.2). Furthermore, line WB had the highest Ab titers to HuSA at d 8 after the start of the heat stress period, and Ab titers were already decreased at d 15 and were further decreased at d 22, whereas the other lines showed similar Ab titers at d 8 and 15, and were decreased at d 22 (data not shown). The effects of heat stress and LPS administration over time are shown in Figure 4.1.

Natural Ab response to HuSA. The total natural Ab response to HuSA during the observation period was affected by a heat × LPS interaction ($P < 0.05$; Table 4.2). Single heat exposure or LPS administration increased natural Ab to HuSA, whereas combined

exposure to heat and LPS decreased natural Ab binding to HuSA compared with the control group. Furthermore, the total natural Ab response to HuSA during the observation period was affected by a heat × time interaction ($P < 0.05$; Table 4.2); at d 5 prior to the start of heat stress, hens in the chamber prepared for heat stress had significantly lower natural Ab binding to HuSA than hens in the chamber prepared for the control temperature. The effects of heat stress and LPS administration over time are shown in Figure 4.2A. No line effects or interactions with line were found.

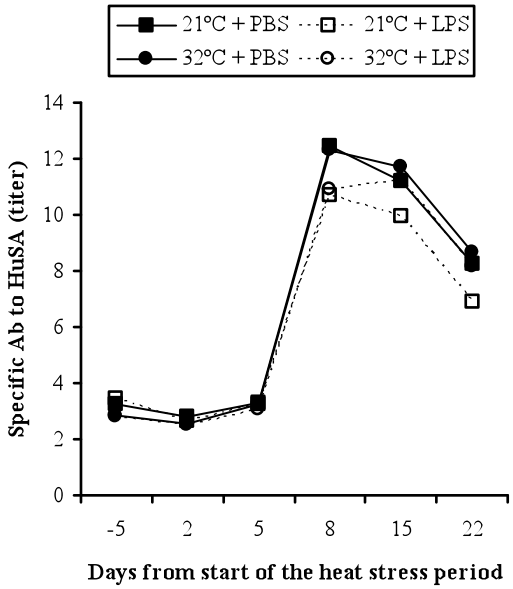

FIGURE 4.1. Effect of heat exposure, administration of *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on the specific antibody (Ab) response to human serum albumin (HuSA) of laying hens (least square mean). Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1 and HuSA was s.c. injected at d 2. Within each treatment group (n = 40 hens per treatment group, except for treatment group 32ºC + LPS, where n = 39), 4 genetically different purebred layer lines, characterized by natural humoral immune competence and survival rate, were equally represented.

NATURAL AB RESPONSE TO KLH. Levels of natural Ab binding to KLH in the sera of hens treated with LPS increased at d 5 after the start of the heat stress period (d 4 after LPS administration), were highest at d 8 after the start of the heat stress period, and were decreased to the baseline level at d 22 after the start of the heat stress period.

TABLE 4.2. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on the average specific and natural antibody (Ab) titer[1] to human serum albumin (HuSA)[2] and natural Ab titer to keyhole limpet haemocyanin (KLH)[3] in 4 purebred layer lines characterized by natural humoral immune competence and survival rate

		Specific Ab[4]	Natural Ab	
Line	Treatment group	(HuSA)	HuSA[4]	KLH[4]
B1	21ºC + PBS	7.10	3.52	3.46
	21ºC + LPS	6.27	4.30	4.51
	32ºC + PBS	7.19	3.40	3.92
	32ºC + LPS	6.77	3.30	4.47
WA	21ºC + PBS	7.16	2.95	2.41
	21ºC + LPS	6.03	3.31	2.74
	32ºC + PBS	7.28	3.36	2.78
	32ºC + LPS	6.57	2.80	3.60
WB	21ºC + PBS	6.43	3.26	3.43
	21ºC + LPS	6.19	3.34	3.68
	32ºC + PBS	6.03	3.52	3.61
	32ºC + LPS	5.84	3.04	3.72
WF	21ºC + PBS	6.80	3.18	4.05
	21ºC + LPS	6.12	3.26	4.44
	32ºC + PBS	6.99	3.36	3.95
	32ºC + LPS	6.61	2.98	4.90
	SEM	0.28	0.32	0.26

[1] Titers were expressed as log2 values of the dilutions that gave extinction closest to E_{max}, where E_{max} represents the highest mean extinction of standard positive serum presented on each flat-bottomed ELISA plate.

[2] Specific Ab titers to HuSA were measured in hens immunized with HuSA (n = 159) and natural Ab titers to HuSA were measured in hens not immunized with HuSA (n = 158).

[3] Natural Ab titers to KLH were measured in all hens (n = 317), irrespective of HuSA immunization.

During the complete observation period, the natural Ab response to KLH was affected by heat ($P < 0.05$), by LPS ($P < 0.0001$), and by line ($P < 0.0001$; Table 4.2). Each of the main effects had an interaction with time [heat × time ($P < 0.01$), LPS × time ($P < 0.0001$), line × time ($P < 0.01$)]. The effects of the treatments over time are shown in Figure 4.2B. Heat increased the natural Ab response to KLH at d 8 and 15 after the start of the heat stress period, whereas LPS increased the natural Ab response to KLH at d 5, 8, and 15. The line ×

TABLE 4.2, CONTINUED. Effect of temperature, Escherichia coli lipopolysaccharide (LPS), or combined exposure to both stressors on the average specific and natural antibody (Ab) titer1 to human serum albumin (HuSA)[2] and natural Ab titer to keyhole limpet haemocyanin (KLH)[3] in 4 purebred layer lines characterized by natural humoral immune competence and survival rate

		Specific Ab[4]	Natural Ab	
Line	Treatment group	(HuSA)	HuSA[4]	KLH[4]
Effects	Line (L)	**	NS	***
		B1, WA ≥ WF ≥ WB		WF ≥ B1 ≥ WB > WA
	Temperature (T)	NS	NS	*
				32ºC > 21ºC
	LPS administration (A)	***	NS	***
		PBS > LPS		LPS > PBS
	Time (Ti)	***	***	***
	L × T	NS	NS	NS
	L × A	NS	NS	NS
	L × Ti	***	NS	**
	T × A	NS	*	NS
	T × Ti	***	*	**
	A × Ti	***	NS	***
	L × T × A	NS	NS	NS
	L × T × Ti	**	NS	NS
	L × A × Ti	NS	NS	NS
	T × A × Ti	NS	NS	NS
	L × T × A × Ti	NS	NS	NS

[4] Values are least square means of Ab titers determined in serum samples collected at d -5, 2, 5, 8, 15, and 22 after the start of the heat stress period. Half of the hens of the heat stress and control groups were injected i.v. with 1 mg/kg of BW of LPS at d 1 after the start of the heat stress period. Half of the LPS- and PBS-treated hens were s.c. immunized with 1 mg of HuSA at d 2 after the start of the heat stress period. The data presented in the table were analyzed by 4-way ANOVA for the effect of line, temperature, LPS administration, time, and their interactions by a repeated measurement procedure using a 'hen nested within line, temperature, and LPS administration' option.

*** P < 0.0001; ** P < 0.01; * P < 0.05; NS = not significant.

time interaction suggests that the lines had different response patterns (data not shown). Line WF had the highest levels of natural Ab binding to KLH during the complete

observation period, whereas line WA had the lowest level of natural Ab binding to KLH (Table 4.2).

HAEMOLYTIC COMPLEMENT ACTIVITY

Results of the effects of single or combined heat exposure and LPS administration on CPW and APW in the 4 layer lines are shown in Table 4.3.

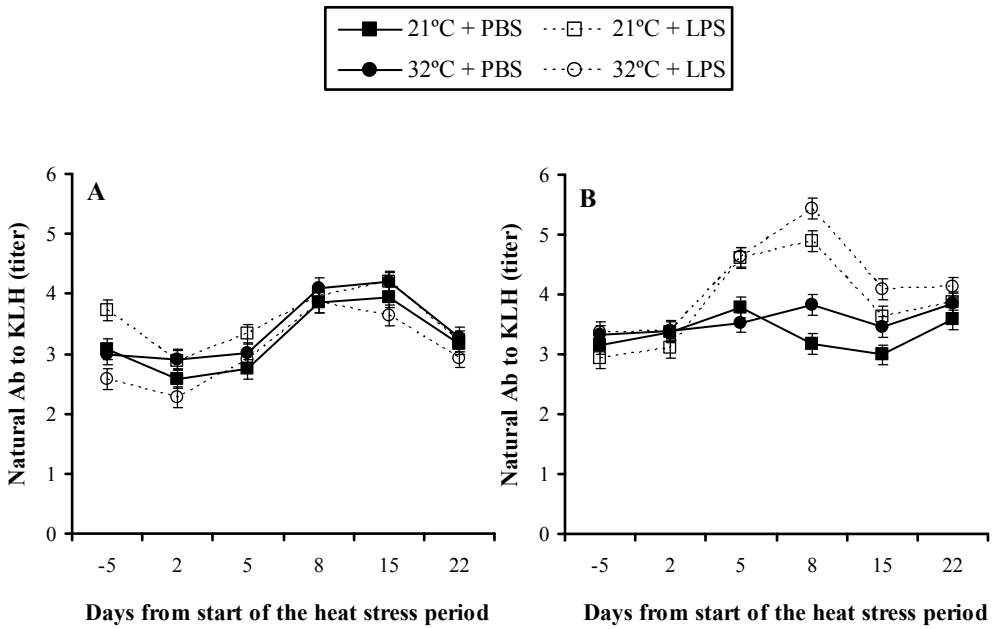

FIGURE 4.2. Effect of heat exposure, administration of *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on natural antibody (Ab) response to [A] human serum albumin (HuSA) or [B] keyhole limpet haemocyanin (KLH) of laying hens (least square mean ± SE). Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1. Within each treatment group (n = 40 hens per treatment group for natural Ab to HuSA, except for treatment group 32ºC + LPS, where n = 38; n = 80 hens per treatment group for natural Ab to KLH, except for treatment group 32ºC + LPS, where n = 77), 4 genetically different purebred layer lines, characterized by natural humoral immune competence and survival rate, were equally represented.

CPW. Activity of CPW was enhanced by LPS administration at d 5 and 8 after the start of the heat stress period (LPS × time interaction; $P < 0.01$), and by heat exposure at d 22 after

the start of the heat stress period (heat × time interaction; $P < 0.0001$). Effects of the treatments over time are shown in Figure 4.3A.

There was a line × time interaction ($P < 0.01$); differences between the lines were found only at d 5 prior to the start of the heat stress period, where line WA had higher CPW activity than line WB (data not shown). No interactions between treatments and lines were found.

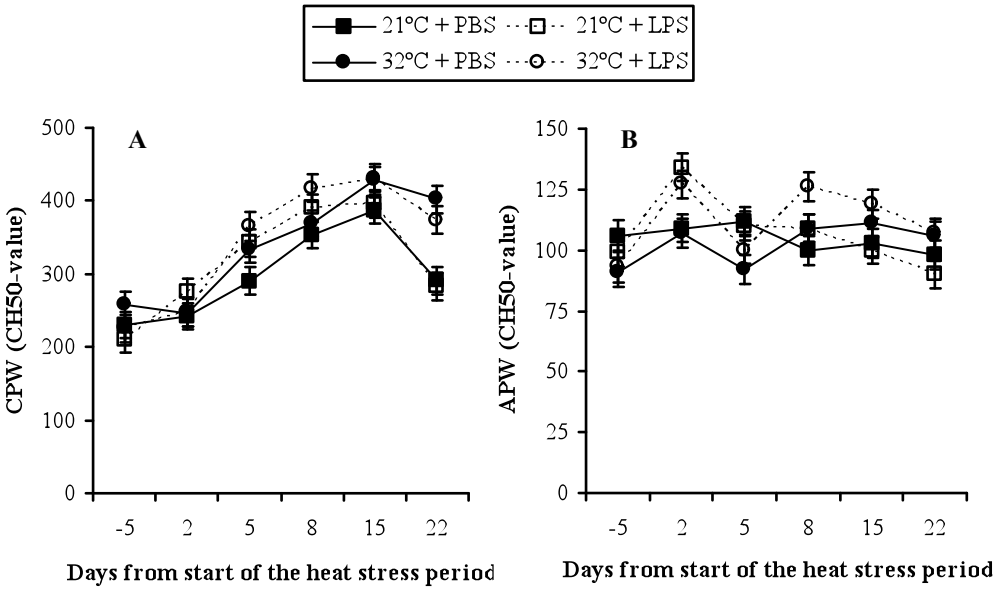

FIGURE 4.3. Effect of heat exposure, administration of *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on activity of the [A] classical complement pathway (CPW) and [B] alternative complement pathway (APW) of laying hens (least square mean ± SE). Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1. Within each treatment group (n = 80 hens per treatment group, except for treatment group 32ºC + LPS, where n = 77), 4 genetically different purebred layer lines, characterized by natural humoral immune competence and survival rate, were equally represented.

APW. Activity of APW was enhanced by LPS administration at d 2 and 8 (LPS × time interaction; $P < 0.01$). Activity of APW was decreased by heat exposure at d 5 after the start of the heat stress period, but was increased by heat exposure at d 8, 15, and 22 after the

Table 4.3. Effect of temperature, Escherichia coli lipopolysaccharide (LPS), or combined exposure to both stressors on average classical complement pathway (CPW)[1] and alternative complement pathway (APW)[1] activity in 4 purebred layer lines characterized by natural humoral immune competence and survival rate

Line	Treatment group	CPW[2]	APW[2]
B1	21ºC + PBS	331	101
	21ºC + LPS	314	95
	32ºC + PBS	382	109
	32ºC + LPS	335	116
WA	21ºC + PBS	296	93
	21ºC + LPS	307	89
	32ºC + PBS	322	93
	32ºC + LPS	357	101
WB	21ºC + PBS	271	87
	21ºC + LPS	306	104
	32ºC + PBS	300	88
	32ºC + LPS	338	107
WF	21ºC + PBS	300	124
	21ºC + LPS	328	133
	32ºC + PBS	350	114
	32ºC + LPS	341	121
	SEM	27	9

[1] CPW and APW activity were measured in all hens (n = 317), irrespective of HuSA immunization.

start of the heat stress period (heat × time interaction; $P < 0.0001$). Effects of the treatments over time are shown in Figure 4.3B.

Line differences were found at each sample moment. Prior to the start of the heat stress period (d -5), line WA had the highest APW activity and line B1 the lowest. At d 2, 5, 8, 15, and 22 after the start of the heat stress period, line WF had the highest APW activity and line WA the lowest APW activity [line × time interaction; $P < 0.0001$ (data not shown)]. Furthermore, there was a line × heat × time interaction ($P < 0.01$), indicating that lines had different APW activity in response to heat stress over time. Most lines showed a comparable pattern. The only line that differed in the response pattern was line WA. Where lines B1, WB, and WF showed an increase in APW activity from d 8 to 15 when exposed to heat, line WA showed a decrease in APW activity when exposed to heat. Irrespective of

TABLE 4.3, CONTINUED. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on average classical complement pathway (CPW)[1] and alternative complement pathway (APW)[1] activity in 4 purebred layer lines characterized by natural humoral immune competence and survival rate

Line	Treatment group	CPW[2]	APW[2]
Effects	Line (L)	NS	***
			WF > B1, WA, WB
	Temperature (T)	*	NS
		32ºC > 21ºC	
	LPS administration (A)	NS	NS
	Time (Ti)	***	***
	L × T	NS	NS
	L × A	NS	NS
	L × Ti	**	***
	T × A	NS	NS
	T × Ti	***	***
	A × Ti	**	***
	L × T × A	NS	NS
	L × T × Ti	NS	**
	L × A × Ti	NS	NS
	T × A × Ti	NS	NS
	L × T × A × Ti	NS	NS

[2] Values are least square means of complement activity determined in serum samples collected at d -5, 2, 5, 8, 15, and 22 after the start of the heat stress period. Half of the hens of the heat stress and control groups were injected i.v. with 1 mg/kg of BW of LPS at d 1 after the start of the heat stress period. Half of the LPS- and PBS-treated hens were s.c. immunized with 1 mg of HuSA at d 2 after the start of the heat stress period. The data presented in the table were analyzed by 4-way ANOVA for the effect of line, temperature, LPS administration, time, and their interactions by a repeated measurement procedure using a 'hen nested within line, temperature, and LPS administration' option.

*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$; NS = not significant.

temperature, line WA showed an increase in APW activity from d 15 to 22, whereas lines B1, WB, and WF showed a decrease (data not shown).

DISCUSSION

In the present study, the effects of single or combined environmental stressors on humoral immune competence in 4 layer lines were investigated. Natural Ab to KLH or HuSA as well as CPW and APW were measured to study the natural humoral immune competence. Immunization with HuSA was done to study the specific humoral immune competence. Exposure to a high temperature for 23 d and single administration of the endotoxin LPS were used as environmental stressors. The administered dose of LPS (i.v. 1 mg/kg of BW) was based on previously reported effects of LPS on poultry (Klasing et al., 1987; Maldonado et al., 2005; Parmentier et al., 2004c), to ensure that the amounts given would result in measurable effects that could be distinguished from effects originating from the intestinal microbiota within poultry kept under normal (LPS-rich) housing conditions. The protein HuSA was used to avoid possible interference with earlier obligatory vaccinations of poultry and the administered dose (s.c. 1 mg) was reported earlier by Maldonado et al. (2005).

In the present study, heat stress and LPS administration acted as two independent stressors. This was illustrated by the lack of interaction between heat stress and LPS administration (except for natural Ab binding to HuSA), and by interactions over time. The response to LPS administration was more pronounced in the first part of the experimental period, whereas the response to heat was more pronounced in the second part of the experimental period. In this respect, it is noteworthy that poultry become refractory to repeated LPS administrations (Korver et al., 1998; Parmentier et al., 2006). Although the present data suggest that single LPS administration was more acute and that the effect of long-term heat stress was more extended, the data do not suggest that recovery from LPS had priority over recovery from heat exposure. Furthermore, our data do not confirm the findings by Dhabhar and Viswanathan (2005) who suggested immune-suppressive effects of chronic stress and immune-enhancing effects of acute stress.

The 4 purebred lines that were used in this study were characterized in a previous experiment (Star et al., 2007a) by high or low natural immune competence and a high or low survival rate. Lines B1 and WF were selected for high natural immune competence, whereas lines WA and WB were selected for low natural immune competence. Ranking of the lines for natural Ab binding to KLH and activity of CPW and APW (data not shown) in the present study was consistent with the previous study. The absence of significant interactions between line and heat stress (except for specific Ab directed to HuSA) or LPS administration on natural and specific humoral immune responses suggests that, regardless of genotype, hens responded similarly to the environmental stressors. However, the lines

differed in their response level and reacted differently over time, indicating the absence of a relationship between genotype and environment. Because the lines were characterized by high or low natural humoral immune competence and a high or low survival rate, we expected that the lines would react differently to environmental stressors. However, the characteristics of the lines were not indicative of the specific and natural immune responses to different stressors.

According to the definition of natural Ab (i.e., that there is no intentional or controllable challenge with the antigen leading to the formation of Ab), KLH and HuSA were chosen to estimate levels of natural Ab. Keyhole limpet haemocyanin is a 'classical' antigen used to measure natural Ab. Keyhole limpet haemocyanin is an exo-antigen that the chickens most probably had not encountered before nor would encounter during their lives. Half of the hens were not immunized with HuSA; therefore, we used these hens to measure natural Ab to HuSA. In the present study, natural Ab to KLH or HuSA were found in all chickens, irrespective of LPS administration, as previously found by Parmentier et al. (2004b) and Star et al. (2007a). To some extent, single administration of LPS enhanced natural Ab titers to HuSA and more pronounced natural Ab titers to KLH. Enhancement of LPS Ab titers after immunization with KLH was reported earlier (Hangalapura et al., 2003,2005; Parmentier et al., 2004c). Lipopolysaccharide administration also enhanced CPW and APW. Administration of LPS in blood activates APW activity almost immediately (Blatteis, 2006), which may be related to the more innate character of APW, as opposed to the more specific character of CPW, although CPW activity was not influenced by HuSA immunization (data not shown). These data suggest that natural Ab and haemolytic complement activity were stimulated by LPS, which confirms the findings of Reid et al. (1997) and Fischer et al. (1997) that the innate immune system is sensitive to microbial components and that it plays an important role in the host defense against bacterial products.

In contrast to the enhancement of natural Ab and complement by LPS administration, specific Ab titers to HuSA were decreased by LPS administration. These results correspond with the reported decreasing effects of LPS on specific Ab titers to bovine serum albumin, KLH, and HuSA (Maldonado et al., 2005; Parmentier et al., 1998,2004c,2006). The dose of LPS and the stimulation of dendritic cells by LPS might affect the balance of Th-1- and Th-2-stimulating cytokines (Boonstra et al., 2003; Langenkamp et al., 2000), and changes in the balance between Th-1 and Th-2 can cause a shift in stimulation of natural or specific immune responses.

In the present study, exposure to heat stimulated natural and specific immune responses. Previous studies on the effect of heat stress on specific immune responses were not consistent. Most studies have described a decreasing effect of heat stress on specific immune responses (Mashaly et al., 2004; McFarlane and Curtis, 1989; Thaxton et al., 1968; Zulkifli et al., 2000), but enhancing effects (Heller et al., 1979) and no effects at all (Donker et al., 1990) were also described. These differences may depend on the length and intensity of heat exposure (Kelley, 1985) or the breed of chicken (Regnier et al., 1980) because genetic variation in heat tolerance is known to exist within species (Mahmoud and Yaseen, 2005), as also indicated by the different response levels of the lines used in the present study. To our knowledge, the enhancing effect of heat stress on natural Ab and complement activity has not been published before. Hangalapura et al. (2003,2004b) observed that the innate immune system of chickens exposed to cold stress (10ºC) responded with enhanced natural Ab responses. These data, as well as the present study, suggest a stimulating effect of high or low temperature on innate immune competence. Hangalapura et al. (2003,2004b), however, found that the innate immune system responded immediately to changes in temperature, whereas in our study the effect of heat stress on innate immune response was extended. Stimulation of the immune system by heat was affected by time, indicating that the effect of heat exposure was not present during the whole experimental period. Our data show that innate and specific immune responses were not affected by heat exposure during the first week, but were mainly affected during the second and third weeks after the start of heat stress.

Our data suggest that LPS and heat stress affected the natural and specific humoral immune competence of laying hens. However, the data also indicate that, based on natural and specific immune competence, hens were able to cope with single or combined heat stress and LPS administration. Furthermore, LPS and heat stress initiated sequential responses over time, with an earlier effect of short-term LPS exposure (within the first and second week) and a later effect of long-term heat exposure (within the second and third week). It has yet to be investigated whether this indicates a priority setting of the chicken between acute or non-acute life-threatening situations or, alternatively, the ability to differentiate between an immunogenic and a physical stressor.

ACKNOWLEDGMENTS

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CHAPTER 5

Effect of single or combined climatic and hygienic stress in four layer lines: 1. Performance

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ABSTRACT

Effects of long-term climatic stress (heat exposure), short-term hygienic stress [lipopolysaccharide (LPS)], or a combination of both challenges on performance of 4 layer lines were investigated. The lines were earlier characterized by natural humoral immune competence and survival rate. At 22 wk of age, 80 hens per line were randomly divided over 2 identical climate chambers and exposed to a constant high temperature (32ºC) or a control temperature (21ºC) for 23 d. Half of the hens housed in each chamber were i.v. injected with LPS at d 1 after the start of the heat stress period. The effect of heat, LPS, or a combined challenge on feed intake, BW, hen-day egg production, egg weight, and egg shell thickness were investigated. Feed intake, BW, hen-day egg production, egg weight, and egg shell thickness were significantly reduced by heat stress. Administration of LPS significantly reduced feed intake, BW (LPS × time interaction), hen-day egg production, and egg weight (LPS × time interaction). Hens were able to recover from LPS administration but did not completely adapt to heat stress. Hens still lost weight, had a lower feed intake and hen-day egg production after 23 d of continuous exposure to heat stress. These data suggest a different nature of short-term LPS exposure versus long-term heat exposure affecting performance parameters of laying hens, and different adaptation mechanisms of hens towards these stressors. Neither natural humoral immune competence nor survival rate, for which the lines had been earlier characterized, were indicative of the response to different stressors. However, significant line × heat interactions were found for feed intake and hen-day egg production, and a line × heat × time interaction for BW, whereas a line × LPS interaction was found for hen-day egg production and a line × LPS × time interaction for BW. The lines had similar response patterns, but differed in response levels, suggesting that some lines were better able to adapt to stressors than others.

Key words: body weight, feed intake, heat stress, hen-day egg production, lipopolysaccharide

Abbreviation key: BW = body weight, LPS = lipopolysaccharide, PBS = phosphate buffered saline

INTRODUCTION

Stress occurs when an animal experiences changes in the environment that stimulate body responses aimed at reestablishing homeostatic conditions (Mumma et al., 2006). Environmental stress include abiotic factors (e.g., climate, temperature, chemical components) and biotic factors (e.g., competition, nutrition, various forms of infectious diseases), which can act independently, but often act synergistically (Bijlsma and Loeschcke, 2005).

Two environmental stressors that might act synergistically are heat stress and microbial challenges. Understanding of the interactions between these two stressors is of interest, first, because both stressors are common in poultry farming and can occur together, and second, because these two stressors represent two physiological drives that utilize common effectors but induce opposite responses (Blatteis, 2000). During heat exposure a rise in body temperature is prevented by panting and vasodilatation of the skin, whereas an infection [induced by lipopolysaccharide (LPS)] is associated with a rise in body temperature (fever), which is achieved by increased metabolic heat production and vasoconstriction of the skin.

Effect of single heat stress in poultry has been frequently studied. Hens raised under moderate climatic circumstances and placed in hot climatic circumstances at the start of lay showed reduced feed intake, BW gain, egg production, egg weight (Mashaly et al., 2004; Njoya and Picard, 1994), and egg shell thickness (Lin et al., 2004c; Usayran et al., 2001) compared with hens kept under moderate circumstances during lay. High environmental temperatures affect not only these performance parameters, but require also various physiological (Koelkebeck and Odom, 1995; Maak et al., 2003) and immunological (Mahmoud and Yaseen, 2005; Mashaly et al., 2004; Thaxton et al., 1968) adaptations of birds.

Lipopolysaccharide, derived from intestinal gram-negative microbiota, is often used as a model antigen to study the animal's susceptibility to (non-specific components of microbiological) pathogens and capability to adapt to immune stressors. Administration of LPS causes sickness symptoms including changes in body temperature (fever), reduced BW gain, and changes in behavior (Adler and DaMassa, 1978; Cheng et al., 2004; Macari et al., 1993; Xie et al., 2000). Effects of microbial challenges, mimicked by LPS, on performance (feed intake, egg production, egg quality) and other physiological responses of adult laying hens are less studied. Besides, effects of combined challenge of heat stress and LPS

administration on performance parameters have, to our knowledge, never been studied in adult laying hens.

In a previous study (Star et al., 2007a) differences in natural humoral immunity were investigated in 12 purebred layer lines. For the present experiment, 4 of the 12 lines were selected, based on high or low natural immune competence and a high or low survival rate. These lines were exposed to the following environmental stressors: heat (climatic stress), LPS (hygienic stress), or combined exposure to heat and LPS. According to Blatteis (2000), we hypothesized that chickens are able to cope with single environmental stressors, but that problems in coping ability occur when chickens are exposed to combined environmental stressors. To evaluate coping ability of different layer lines, effects of climatic and hygienic stress on performance (e.g., feed intake, BW, hen-day egg production, egg weight, and egg shell thickness) and physiological responses were investigated. The current paper will focus on the effect of different stressors on performance of 4 different layer lines. First, we investigated responses, as expressed in performance parameters, by single exposure to high temperature or LPS administration, or combined exposure to both stressors. Second, we investigated if different chicken lines had different responses in performance parameters when exposed to climatic or hygienic stress, and whether one line was better able to withstand stress than another line. In an accompanying paper (Star et al., 2008b), effects of exposure to climatic and hygienic stress on physiological responses in these layer lines is reported.

MATERIALS AND METHODS

Chickens, housing, and feed

Four purebred layer lines (Hendrix Genetics, Boxmeer, the Netherlands) were used: 3 White Leghorn lines (WA, WB, and WF), and 1 Rhode Island Red line (B1). These lines were characterized by a low or high survival rate and low or high natural humoral immune competence as determined in a previous study (Star et al., 2007a). Line B1 was characterized by a low survival rate and high natural humoral immune competence, line WA was characterized by a high survival rate and low natural humoral immune competence, line WB was characterized by a low survival rate and low natural humoral immune competence, and line WF was characterized by a high survival rate and high natural humoral immune competence.

At 22 wk of age, 80 hens per line (320 in total) were transported from a housing facility at Hendrix Genetics to 2 identical climate respiration chambers at Wageningen University.

In each climate chamber, 40 hens per line were individually housed in battery cages (45 cm height × 40 cm depth × 24 cm width). Lines were randomly divided over cages. Hens were fed a standard commercial phase 1 diet (15.9% crude protein, 3.9% crude fiber, and 11.8 MJ of ME/kg). At 22 wk of age, hens were kept under a 13L:11D light scheme. In each of the following 2 wk, the light period was increased with 1 h. At the start of the experimental period (at 24 wk of age), hens were kept under a 15L:9D light scheme until the end of the experimental period (27 wk of age). Hens received routine vaccinations to Marek's disease (d 1), Newcastle disease (wk 2, 6, 12, 15), infectious bronchitis (d 1, wk 2, 10, 12, 15), infectious bursal disease (wk 3, 15), fowl pox (wk 15), and avian encephalomyelitis (wk 15). Beak trimming was not performed.

TABLE 5.1. Experimental design[1]

Treatment group	Temperature	LPS[2]	# animals	# animals per line
21ºC + PBS	21ºC	-	80	20
21ºC + LPS	21ºC	+	80	20
32ºC + PBS	32ºC	-	80	20
32ºC + LPS	32ºC	+	80	20

[1] The experiment consisted of 4 treatment groups, which were exposed to a temperature of 21 or 32ºC during 23 d, and were i.v. injected with *Escherichia coli* lipopolysaccharide (LPS) at d 1 after the start of the heat stress period. Within each treatment group, 4 genetically different purebred layer lines, characterized by natural humoral immune competence and survival rate, were equally represented. Line B1 was characterized by a low survival rate and high natural humoral immune competence, line WA was characterized by a high survival rate and low natural humoral immune competence, line WB was characterized by a low survival rate and low natural humoral immune competence, and line WF was characterized by a high survival rate and high natural humoral immune competence.

[2] + = hens injected with 1 mg/kg of BW of LPS; - = hens injected with PBS.

EXPERIMENTAL DESIGN

After an adaptation period of 12 d (temperature maintained at 21ºC), hens in the first climate chamber were exposed to acute heat stress. Within approximately 1 h, the temperature in this chamber was increased from 21 to 32ºC, and was maintained at 32ºC for the following 23 d. In the second chamber, the (control) temperature was maintained at 21ºC. At d 1 after the start of the heat stress period, half of the hens in the heat treatment and half of the control hens were i.v. injected with 1 mg/kg of BW of *Escherichia coli* LPS (serotype O55:B5, Sigma Chemical Co., St. Louis, MO). The remaining hens received a placebo treatment of PBS. An overview of the experimental design is given in Table 5.1.

The Institutional Animal Care and Use Committee of Wageningen University approved the experimental protocol.

Performance

During both adaptation and experimental period, feed and water were available ad libitum. Individual feed intake was recorded daily during adaptation and experimental period. BW was measured weekly at d 5 before the start of heat stress and at d 2, 8, 15, and 22 during heat stress. Weekly BW gain was calculated compared with the BW measured at d 5 before the start of heat stress. Egg number was recorded daily during adaptation and experimental period. Egg weight was recorded weekly at d 3 before the start of heat stress and at d 4, 11, and 18 during heat stress. Shell thickness (without inner and outer shell membranes) was measured at the middle part of the egg using a micrometer (Mitutoyo, Miyazaki, Japan), once at the end of the experimental period (d 23). Mortality was registered.

Statistical analysis

Differences in feed intake, hen-day egg production, and egg shell thickness were analyzed by a 3-way ANOVA for the effect of line, temperature, LPS administration, and their interactions. Daily measured feed intake and hen-day egg production were, after primary analyses of the daily measurements, divided in 3 periods; P1 is the adaptation period (d -11 to 0), P2 is the experimental period where temperature and LPS administration were of influence (d 1 to 7), and P3 is the experimental period where only temperature was of influence (d 8 to 22). Statistical analyses were done for each period, and no statistical analyses were done between periods. Differences in BW and egg weight were analyzed by a 4-way ANOVA for the effect of line, temperature, LPS administration, time, and their interactions by a repeated measurement procedure using a 'hen nested within line, temperature, and LPS administration' option.

Mean differences among lines and treatments were tested with Bonferroni's test. All analyses were carried out using SAS (SAS Institute, 2004). Effects were considered significant at $P < 0.05$.

RESULTS

MORTALITY

Within 1 d after LPS administration 3 hens died (1 of line B1 and 2 of line WA). These hens were exposed to the combined challenge of LPS and heat. All other hens survived the experimental period.

FEED INTAKE

Main effects of exposure to heat, LPS administration, or combined exposure to heat and LPS are shown in Figure 5.1 and will be described first, whereas more detailed results per line are given in Table 5.2 and will be described after the main effects.

Figure 5.1 shows that exposure to heat reduced feed intake during the whole experimental period (P2 and P3; $P < 0.0001$), whereas the effect of LPS was only noticeable during the first week after administration (P2; $P < 0.0001$).

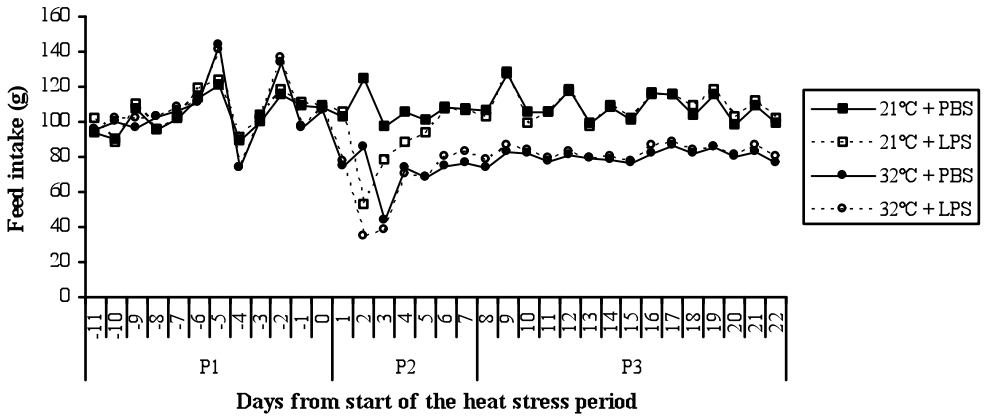

FIGURE 5.1. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on daily feed intake (LSMean) of laying hens. Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1 after the start of the heat stress period. The experiment was divided in 3 periods; P1 is the adaptation period (d -11 to 0), P2 is the experimental period where both temperature and LPS administration were of influence (d 1 to 7), and P3 is the experimental period where only temperature was of influence (d 8 to 22).

During P2, the control group had an average feed intake of 106.5 g, whereas hens injected with LPS had an average feed intake of 90.2 g. Hens exposed to heat stress or to

combined heat and LPS had an average feed intake of 70.9 and 64.7 g, respectively. There was a significant heat × LPS interaction during P2 ($P < 0.01$), indicating that combined exposure had less effect on feed intake than the sum of the individual effects.

Heat stress significantly reduced feed intake during P3, with a difference of 27.1 g feed intake between hens exposed to 21 or 32ºC.

Feed intake was affected by line during P1, P2, and P3 ($P < 0.0001$, $P < 0.05$, and $P < 0.01$, respectively). Significant line × heat interaction was found during P2 and P3 ($P < 0.05$ and $P < 0.0001$, respectively). Decreases in feed intake between heat-stressed group (32ºC + PBS) and control group (21ºC + PBS) were 42.9, 41.1, 28.5, and 29.7 g for line B1, WA, WB, and WF, respectively, during P2. Decreases in feed intake between heat-stressed group (32ºC + PBS and 32ºC + LPS) and control group (21ºC + PBS and 21ºC + LPS) were 36.9, 24.6, 23.8, and 23.1 g for line B1, WA, WB, and WF, respectively, during P3. Although each line had a reduced feed intake during heat exposure, the feed intake of the Rhode Island Red line B1 seemed to be more influenced by heat stress than the feed intake of the White Leghorn lines.

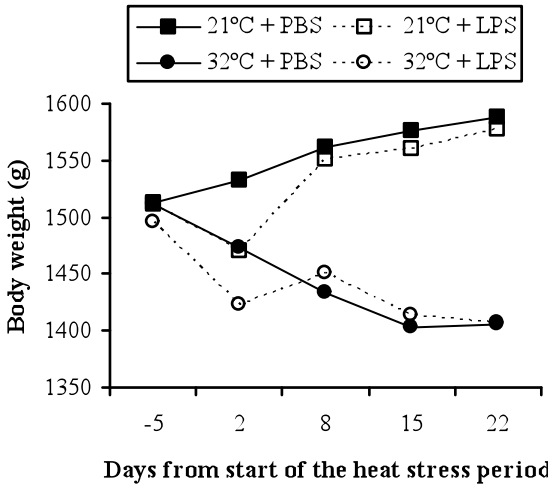

FIGURE 5.2. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on average BW (LSMean) of laying hens at d -5, 2, 8, 15, and 22 after the start of the heat stress period. Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1 after the start of the heat stress period.

BODY WEIGHT

Main effects of exposure to heat, LPS administration, or combined exposure to heat and LPS are shown in Figure 5.2 and will be described first, whereas more detailed results per line are given in Table 5.4 and will be described after the main effects.

Figure 5.2 shows that exposure to heat decreased BW during the whole experimental period (heat × time interaction; $P < 0.0001$), whereas the effect of LPS administration was only noticeable at d 2 after start of the heat stress period (LPS × time interaction; $P < 0.0001$). At d 8 after the start of heat stress (i.e., d 7 after LPS administration) hens were recovered from the LPS administration. They gained weight and had a comparable BW as their counterparts housed at 21 or 32ºC (heat × LPS × time interaction; $P < 0.05$).

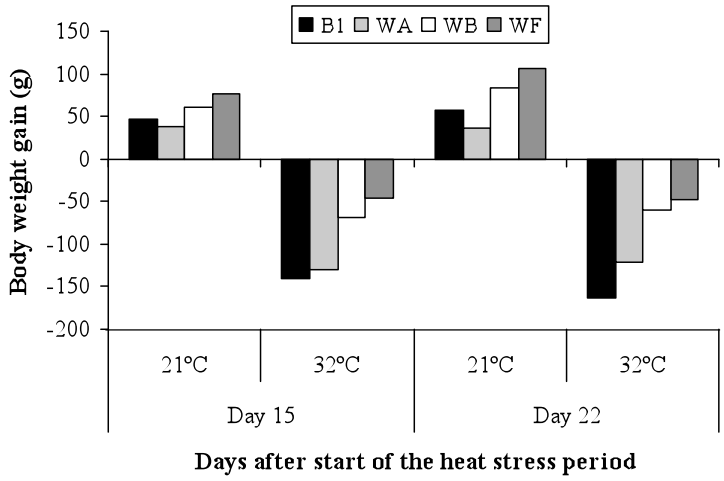

FIGURE 5.3. Body weight gain (LSMean) for the 4 genetically different purebred layer lines housed at 21 or 32ºC at d 15 and 22 after the start of the heat stress period compared with d 5 before the start of heat stress. Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0.

Before start of the heat stress period, line B1, WA, WB, and WF had a mean BW of 1698, 1510, 1397, and 1428 g, respectively. During the complete experiment the Rhode Island Red line B1 was heavier than the White Leghorn lines ($P < 0.0001$). The lines showed different patterns of BW gain under the given circumstances, illustrated by a line × heat × time interaction ($P < 0.05$; Figure 5.3) and a line × LPS × time interaction ($P < 0.05$). On the one hand, lines B1 and WA gained lesser weight in the control chamber (BW gain

TABLE 5.2. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on average feed intake (g) in 4 genetically different purebred layer lines before challenge (d -11 to 0; P1), during LPS influence (in combination with or without heat stress; d 1 to 7; P2), and during heat stress alone (d 8 to 22; (P3)

		Feed intake		
Line	Treatment group	P1	P2	P3
B1	21ºC + PBS	105.4	109.2[a]	111.2[a]
	21ºC + LPS	101.3	84.8[b]	111.8[a]
	32ºC + PBS	108.6	66.3[c]	72.4[b]
	32ºC + LPS	107.9	60.0[c]	77.0[b]
WA	21ºC + PBS	113.4	108.6[a]	105.2[a]
	21ºC + LPS	116.1	92.3[b]	109.1[a]
	32ºC + PBS	110.8	67.5[c]	80.2[b]
	32ºC + LPS	116.6	62.3[c]	84.9[b]
WB	21ºC + PBS	99.9	103.8[a]	105.6[a]
	21ºC + LPS	105.7	89.8[b]	104.8[a]
	32ºC + PBS	102.0	75.3[c]	83.0[b]
	32ºC + LPS	102.9	66.8[c]	79.8[b]
WF	21ºC + PBS	98.5	104.4[a]	111.3[a]
	21ºC + LPS	102.4	94.0[a]	108.5[a]
	32ºC + PBS	101.1	74.7[b]	85.5[b]
	32ºC + LPS	101.6	68.7[b]	88.5[b]
	SEM	2.82	2.57	2.38

[a-c] Means with different superscript within treatment group of each line differ significantly ($P < 0.05$).

of 57 and 37 g, respectively) compared with lines WB and WF (BW gain of 84 and 106 g, respectively). On the other hand, lines B1 and WA lost most weight in the heat stress chamber (BW loss of 163 and 121 g, respectively) compared with lines WB and WF (BW loss of 60 and 48 g, respectively).

HEN-DAY EGG PRODUCTION

Main effects of exposure to heat, LPS administration, or combined exposure to heat and LPS are shown in Figure 5.4 and will be described first, whereas more detailed results per line are given in Table 5.3 and will be described after the main effects.

TABLE 5.2, CONTINUED. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on average feed intake (g) in 4 genetically different purebred layer lines before challenge (d -11 to 0; P1), during LPS influence (in combination with or without heat stress; d 1 to 7; P2), and during heat stress alone (d 8 to 22; P3)

		Feed intake		
Line	Treatment group	P1	P2	P3
Effects	Line (L)	***	*	**
		WA > B1, WB, WF	WF ≥ WB, WA ≥ B1	WF ≥ WA ≥ WB, B1
	Temperature (T)	NS	***	***
			21ºC > 32ºC	21ºC > 32ºC
	LPS administration (A)	NS	***	NS
			PBS > LPS	
	L × T	NS	*	***
	L × A	NS	NS	NS
	T × A	NS	**	NS
	L × T × A	NS	NS	NS

*** P < 0.0001; ** P < 0.01; * P < 0.05; NS = not significant.

Heat exposure and LPS administration influenced hen-day egg production (Figure 5.4). Exposure to heat reduced hen-day egg production during the complete experimental period [P2 ($P < 0.01$) and P3 ($P < 0.0001$)]. Hens exposed to heat had a hen-day egg production of 83.8% and 83.6% compared with a hen-day egg production of 93.2% and 93.0% in the control group during P2 and P3, respectively. Administration of LPS decreased hen-day egg production during P2 ($P < 0.0001$) and increased hen-day egg production during P3 ($P < 0.05$). Single LPS administration or combined with heat stress gave comparable reductions in hen-day egg production during P2 (hen-day egg production of 55.0% and 51.4%, respectively).

After LPS administration, hen-day egg production was 43.7, 51.5, 54.6, and 62.9% for line B1, WA, WB, and WF during P2, respectively, which was a significant lower hen-day egg production then their PBS treated counterparts (line × LPS interaction; $P < 0.01$). Decrease in hen-day egg production caused by heat stress was less than 10% and was not significant in each of the White Leghorn lines. Rhode Island Red line B1 had a hen-day egg production of 96.4 and 73.6% when housed by 21 and 32ºC, respectively (line × heat interaction; $P < 0.01$).

Decreases in hen-day egg production between heat-stressed and control group during P3 were 24.0, 4.3, 0.0, and 1.1% for line B1, WA, WB, and WF, respectively. Line B1 was the only line in which heat-stressed hens differed in hen-day egg production from non-heat stressed hens (line × heat interaction; $P < 0.0001$). Interestingly, this is mainly caused by heat-stressed hens that did not receive LPS.

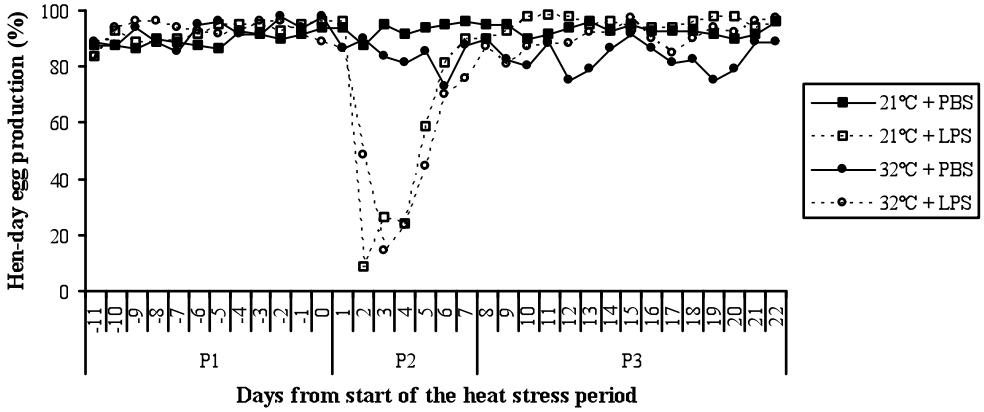

FIGURE 5.4. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on hen-day egg production of laying hens. Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1 after the start of the heat stress period. The experiment was divided in 3 periods; P1 is the adaptation period (d -11 to 0), P2 is the experimental period where both temperature and LPS administration were of influence (d 1 to 7), and P3 is the experimental period where only temperature was of influence (d 8 to 22).

EGG WEIGHT

Average egg weight per line per treatment is given in Table 5.4. Each of the main effects had an interaction with time [heat × time ($P < 0.0001$), LPS × time ($P < 0.0001$), line × time ($P < 0.05$)]. Heat stress reduced egg weight at d 4, 11, and 18 after the start of heat stress. Administration of LPS reduced egg weight at d 4 after the start of heat stress. The line × time interaction suggests that the lines had a different response pattern (data not shown). At the end of the experiment, hens housed in the heat chamber laid eggs with, on average, a 5.2 g lower weight compared with hens housed in the control chamber. Line B1 and WB had the largest difference in egg weight; 5.8 g (10.8%) and 5.5 g (10.3%), respectively. The difference in line WB is mainly caused by an increase in egg weight in the control chamber (+ 4.5 g), whereas the difference in line B1 is caused by a lower increase in egg weight in

the control chamber (+ 3.4 g) and a stronger decrease in egg weight in the heat chamber (-2.4 g). Line WA and WF had an increase in egg weight in the control chamber of 4.1 and 4.2 g, respectively, and a decrease in egg weight in the heat chamber of 1.0 and 0.5 g, respectively, with a total difference of 8.8 and 8.6%, respectively.

EGG SHELL THICKNESS

Egg shell thickness was measured at the end of the heat stress period (d 23). Shell thickness was affected by line ($P < 0.0001$) and by heat stress ($P < 0.0001$; Table 5.4). Line WF had the thickest egg shell in both control and heat chamber, 385 μm and 357 μm, respectively, whereas line WB had the thinnest egg shell in both control and heat chamber, 322 μm and 296 μm, respectively. Difference in egg shell thickness between control and heat chamber was comparable among lines. Line B1, WA, WB, and WF had a difference in shell thickness of 9.7, 9.3, 8.1, and 7.0%, respectively, between control and heat chamber.

DISCUSSION

Effects of combined challenge of heat stress and LPS on performance parameters has, to our knowledge, never been studied in adult laying hens. Understanding of the interactions between these stressors is of interest, first, because both stressors are common in poultry farming and can occur together, and second, because these stressors represent two physiological drives that utilize common effectors but induce opposite responses (Blatteis, 2000). Studies done in rats (Kamerman et al., 2001; Kluger et al., 1997) and rabbits (Blatteis, 2000) are based on short-term exposure to heat stress (maximum of 24 h) with LPS administration at the start of the heat stress or 4 to 48 h after end of heat exposure. Blatteis (2000) mentioned that single heat stress or LPS administration did not result in mortality, whereas combined exposure to heat and LPS resulted in 16.7% mortality. In the present study, 3.8% (3/80) hens died due to combined exposure to heat and LPS, and none of the hens died by exposure to the single stressors.

In the present study, heat stress reduced feed intake, BW, hen-day egg production, egg weight, and egg shell thickness. Hens did not completely adapt to heat stress, because hens were still losing weight and had a lower feed intake and lower hen-day egg production after 23 d of continuous exposure to heat stress. Although there was no replication of the heat stress environment (possible confounded results), these findings confirm earlier studies (Lin et al., 2004c; Mashaly et al., 2004; Njoya and Picard, 1994; Usayran et al., 2001). Maak et al. (2003), however, did not find differences in BW and hen-day egg production when birds were exposed to permanent heat stress (from chick to 68 wk of age). In the

TABLE 5.3. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on hen-day egg production (%) in 4 genetically different purebred layer lines before challenge (d -11 to 0; P1), during LPS influence (in combination with or without heat stress; d 1 to 7; P2), and during heat stress alone (d 8 to 22; P3)

		Egg production		
Line	Treatment group	P1	P2	P3
B1	21ºC + PBS	83.8	96.4[a]	97.0[a]
	21ºC + LPS	89.6	52.1[c]	95.7[a]
	32ºC + PBS	95.8	73.6[b]	66.0[b]
	32ºC + LPS	92.5	35.3[c]	80.1[ab]
WA	21ºC + PBS	90.4	93.6[a]	92.0
	21ºC + LPS	94.6	50.7[b]	96.3
	32ºC + PBS	95.4	88.6[a]	89.3
	32ºC + LPS	96.7	52.4[b]	90.4
WB	21ºC + PBS	95.8	94.3[a]	96.7
	21ºC + LPS	93.4	56.4[b]	94.0
	32ºC + PBS	94.6	88.6[a]	93.7
	32ºC + LPS	95.4	52.6[b]	95.7
WF	21ºC + PBS	87.5	88.6[a]	86.3
	21ºC + LPS	90.0	60.7[c]	95.3
	32ºC + PBS	82.9	84.3[ab]	85.3
	32ºC + LPS	86.3	65.0[bc]	94.3
	SEM	3.16	4.08	3.57

[a-c] Means with different superscript within treatment group of each line differ significantly ($P < 0.05$).

present study, reduction in feed intake during heat stress may cause a decrease in BW, hen-day egg production, and egg weight, indicating a negative nutrient balance of heat-stressed laying hens (Mashaly et al., 2004). Decrease in egg shell thickness at high temperature might be due to disturbance of shell formation (Lin et al., 2004c).

Administration of LPS causes sickness behavior. Sickness behavior includes non-specific symptoms of infection [e.g., weakness, malaise, listlessness, and fever (Dantzer, 2001)]. During the first 24 h after LPS administration chickens were very inactive, illustrated by an increase in sitting behavior and a decrease of standing, feeding, drinking, and moving behavior (Cheng et al., 2004). In the present study, sickness was not studied. Sickness was, however, indirectly measured by feed intake and hen-day egg production. Feed intake was

TABLE 5.3, CONTINUED. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on hen-day egg production (%) in 4 genetically different purebred layer lines before challenge (d -11 to 0; P1), during LPS influence (in combination with or without heat stress; d 1 to 7; P2), and during heat stress alone (d 8 to 22; P3)

		Egg production		
Line	Treatment group	P1	P2	P3
Effects	Line (L)	**	**	**
		WB, WA ≥ B1 ≥ WF	WF, WB ≥ WA ≥ B1	WB, WA ≥ WF ≥ B1
	Temperature (T)	NS	**	***
			21ºC > 32ºC	21ºC > 32ºC
	LPS administration (A)	NS	***	*
			PBS > LPS	LPS > PBS
	L × T	NS	**	***
	L × A	NS	**	NS
	T × A	NS	NS	NS
	L × T × A	NS	NS	NS

*** P < 0.0001; ** P < 0.01; * P < 0.05; NS = not significant.

significantly reduced by LPS administration, and it took at least 5 d to recover from this challenge. Sickness was well illustrated by hen-day egg production. Within hours after LPS administration almost all challenged hens laid a shell-less egg. Thereafter it took also 5 d to recover hen-day egg production. Sickness and reduction in feed intake caused a decrease in BW 1 d after LPS administration, which confirms earlier findings of Cheng et al. (2004) and Parmentier et al. (1998). One week after LPS administration, hens were recovered, illustrated by a comparable feed intake, BW, and hen-day egg production to their PBS-treated counterparts, suggesting an acute but short-term effect of LPS administration.

Hens exposed to combined heat and LPS administration had a lower feed intake during the first week after the start of the heat stress period than hens exposed to heat or LPS. The strong reduction in feed intake after LPS administration combined with heat stress resulted in the lowest BW for this treatment group at d 2 after the start of the heat stress period. At d 8, 15, and 22, however, the effect of combined heat exposure and LPS administration was less negative on BW than single exposure to heat. This indicates that the combined treatment has a stronger, although not additive, effect in the first week. Hereafter, the hens were recovered from LPS administration and were able to maintain a constant feed intake

TABLE 5.4. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on BW (g), egg weight (g), and shell thickness (μm) in 4 genetically different purebred layer lines

Line	Treatment group	Body weight[1]	Egg weight[1]	Shell thickness[2]
B1	21ºC + PBS	1725.1	52.3^{a}	363^{ab}
	21ºC + LPS	1684.8	50.1^{ab}	378^{a}
	32ºC + PBS	1635.9	47.4^{b}	334^{b}
	32ºC + LPS	1608.4	47.3^{b}	337^{b}
WA	21ºC + PBS	1526.7^{ab}	53.5^{a}	341^{ab}
	21ºC + LPS	1557.0^{a}	52.9^{ab}	348^{a}
	32ºC + PBS	1395.7^{ab}	49.3^{c}	312^{b}
	32ºC + LPS	1427.6^{ab}	50.1^{bc}	313^{b}
WB	21ºC + PBS	1434.2	52.0^{a}	319^{ab}
	21ºC + LPS	1438.0	51.1^{ab}	325^{a}
	32ºC + PBS	1366.0	48.5^{b}	299^{ab}
	32ºC + LPS	1350.3	48.4^{b}	293^{b}
WF	21ºC + PBS	1529.5^{a}	52.4^{a}	397^{a}
	21ºC + LPS	1456.9^{ab}	51.8^{ab}	373^{ab}
	32ºC + PBS	1384.3^{ab}	48.7^{bc}	351^{b}
	32ºC + LPS	1359.4^{ab}	47.9^{c}	362^{b}
	SEM	26.9	0.65	6.60

$^{a-c}$ Means with different superscript within treatment group of each line differ significantly ($P < 0.05$).

[1] Differences in BW and egg weight were analyzed by a 4-way ANOVA for the effect of line, temperature, LPS administration, time, and their interactions by a repeated measurement procedure using a 'hen nested within line, temperature, and LPS administration' option. Values are least square means of BW and egg weight measured at d -5, 2, 8, 15, 22 and d -3, 4, 11, 18 after the start of the heat stress period, respectively.

[2] Differences in egg shell thickness (at d 23 after start of the heat stress period) were analyzed by a 3-way ANOVA for the effect of line, temperature, LPS administration, and their interactions.

comparable with feed intake of hens exposed to single heat stress. Feed intake was, however, too low to maintain BW under high temperature treatment.

In the present study, 4 purebred layer lines were used. Lines were characterized by natural humoral immune competence and survival rate as described in a previous study (Star et al., 2007a). Neither natural humoral immune competence nor survival rate was indicative for the physiological and reproductive responses of the 4 lines to the different environmental stressors. Although the lines showed similar response patterns, they differed

TABLE 5.4, CONTINUED. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on BW (g), egg weight (g), and shell thickness (μm) in 4 genetically different purebred layer lines

Line	Treatment group	Body weight[1]	Egg weight[1]	Shell thickness[2]
Effects	Line (L)	***	***	***
		B1 > WA ≥ WF ≥ WB	WA > WF, WB, B1	WF > B1 > WA >WB
	Temperature (T)	***	***	***
		21ºC > 32ºC	21ºC > 32ºC	21ºC > 32ºC
	LPS administration (A)	NS	NS	NS
	Time (Ti)	***	***	
	L × T	NS	NS	NS
	L × A	NS	NS	NS
	L × Ti	***	*	
	T × A	NS	NS	NS
	T × Ti	***	***	
	A × Ti	***	***	
	L × T × A	NS	NS	*
	L × T × Ti	*	NS	
	L × A × Ti	*	NS	
	T × A × Ti	*	NS	
	L × T × A × Ti	NS	NS	

*** P < 0.0001; ** P < 0.01; * P < 0.05; NS = not significant.

in response levels. Rhode Island Red line B1 had the strongest reduction in feed intake, BW, and hen-day egg production during heat stress compared with the White Leghorn lines. Marsden et al. (1987) found a stronger reduction in feed intake and BW in a Brown layer type compared to a White layer type when exposed to a temperature of 30ºC, but they did not find a difference in egg production between the lines. In the present study, differences in performance were also found between the White Leghorn lines, although these differences were smaller than between the Rhode Island Red type and White Leghorn types. Line WA had a stronger decrease in BW and a lower hen-day egg production during heat stress than line WB and WF. Hester et al. (1996b) used 3 White Leghorn lines: a line selected for high group productivity and survivability, a random bred control line, and a

commercial line. Compared with hens of the control line and commercial line, hens of the high selection line had an improved adaptability to high temperature conditions. Hester et al. (1996a,b,c) concluded from their studies that, from criteria used to evaluate stress (e.g., physiological and immunological parameters), egg production and mortality provided the best evidence for adaptability to stress. Our data, from the previous and current study, also suggest that some lines are better able to cope with environmental stressors than other lines based on egg production and mortality. Line B1 had a high mortality rate under commercial circumstances (previous study) and showed a decline in hen-day egg production by exposure to high temperatures. Line WA had a decline in production at the end of the laying period (60 to 69 wk of age) under commercial circumstances (previous study; unpublished data) and had more problems with keeping up production under heat stress than the other White Leghorn lines. Line WB and WF were able to maintain a high hen-day egg production under heat stress. However, line WF was a better survivor under commercial circumstances than line WB (previous study), which makes line WF a more robust line.

Under the given circumstances (temperature of 21 or 32ºC with or without injection of 1 mg/kg of BW of LPS), the results of this study indicate that heat exposure and LPS administration were independent stressors. Hens were able to recover from LPS administration, but did not completely adapt to heat stress, since hens were still losing weight, had a lower feed intake and hen-day egg production after 23 d of continuous exposure to heat stress. The 4 purebred layer lines had similar response patterns, but differed in response levels (especially for hen-day egg production and feed intake), suggesting that some lines were better able to adapt to stressors than other lines. Neither natural humoral immune competence nor survival rate, on which bases the lines were characterized, was indicative for the stress response to different stressors.

Finally, studies in broiler chickens indicated that neonatal heat exposure at 5 d of age reduced mortality (Arjona et al., 1990; Yahav and Hurwitz, 1996) and improved performance and thermo tolerance later in life (Yahav and Plavnik, 1999) by elevation of the thermoregulatory set point (Tzschentke, 2004). In the present study, adult laying hens were exposed to acute, long-term heat stress. Early-age thermal conditioning of layer chicks might improve thermo tolerance and might prevent strong reductions in feed intake, BW, and hen-day egg production during thermal challenge later in life.

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CHAPTER 6

Effect of single or combined climatic and hygienic stress in four layer lines: 2. Endocrine and oxidative stress responses

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ABSTRACT

Effects of long-term climatic stress (heat exposure), short-term hygienic stress [lipopolysaccharide (LPS)], or combined exposure to these stressors on endocrine and oxidative stress parameters of 4 layer lines (B1, WA, WB, and WF) were investigated. The lines were earlier characterized for natural humoral immune competence and survival rate. Eighty hens per line were randomly divided over 2 identical climate chambers and exposed to constant high temperature (32ºC) or a control temperature (21ºC) for 23 d. Half of the hens housed in each chamber were i.v. injected with LPS at d 1 after the start of the heat stress period. The effect of heat, LPS, or combined exposure on plasma levels of corticosterone, 3,5,3'-triiodothyronine (T3), glucose, uric acid (UA), and thiobarbituric acid reacting substances (TBARS) were investigated. Except for UA, there were no interactions between heat stress and LPS administration. Heat stress enhanced levels of corticosterone, glucose, and TBARS, whereas levels of T3 and UA were decreased. The T3 levels, however, were enhanced by LPS administration, whereas levels of UA were decreased. Administration of LPS had no effect on levels of corticosterone and TBARS. Because both stressors caused a reduction in feed intake, it is assumed that changes in most of the plasma levels of the endocrine and oxidative stress parameters are related with the reduction in feed intake. Neither natural humoral immune competence nor survival rate, for which the lines have been characterized, was indicative for the endocrine and oxidative stress responses to different stressors. The present data suggest that hens were able to cope with single or combined heat stress and LPS administration and that heat stress and LPS administration acted like 2 independent stressors. Furthermore, the 4 layer lines differed in response patterns and response levels; line WB was physiologically most sensitive to environmental changes.

Key words: corticosterone, glucose, heat stress, lipopolysaccharide, 3,5,3'-triiodothyronine

Abbreviation key: BW = body weight, LPS = lipopolysaccharide, PBS = phosphate buffered saline, T3 = 3,5,3'-triiodothyronine, TBARS = thiobarbituric acid reacting substances, UA = uric acid

INTRODUCTION

Various physiologic and metabolic changes occur when chickens are exposed to heat (Gonzalez-Esquerra and Leeson, 2006). To compensate for physiologic disturbances of the body by heat stress, more glucocorticoid is released. Glucocorticoids, as the final effectors of the hypothalamic-pituitary-adrenal axis, participate in the control of whole body homeostasis and the response of the organism to stress (Lin et al., 2004a). The major adrenal glucocorticoid hormone in birds is corticosterone (Carsia and Harvey, 2000). Changes in corticosterone levels occur as a function of environmental stimuli (Korte et al., 2005). Furthermore, plasma concentrations of 3,5,3'-triiodothyronine (T3) are related to environmental temperature (Yahav et al., 1996), and levels fall immediately after heat exposure (Uni et al., 2001). The importance of the thyroid gland in adaptation to heat stress is related to the central role that thyroid hormones play in regulation of metabolic rate of birds (Decuypere and Kühn, 1988; Kühn et al., 1984). A more common reaction to (different) stressors is the increase of free radicals in the body: oxidative stress. Oxidative stress can be induced by acute heat stress, resulting in elevated levels of thiobarbituric acid reacting substances (TBARS, a read-out for lipid peroxidation, the most extensively studied consequence of free radical attack; Lin et al., 2006a). Furthermore, the antioxidant uric acid (UA) is an end product of protein metabolism, and levels are affected by stress. Besides the protein metabolism, also gluconeogenesis is influenced by stress. Increased circulating glucocorticoids, on the one hand, induce gluconeogenesis and, on the other hand, suppress glucose uptake of the cells (Munck et al., 1984), helping to maintain or elevate plasma glucose levels.

Lipopolysaccharide (LPS) is often used as a model antigen to study the susceptibility of the animal to (non-specific components of microbiological) pathogens. Lipopolysaccharide is an acute inflammatory stimulus, and challenge with LPS stimulates the synthesis and release of glucocorticoids, inducing a rapid, short-lived increase in plasma corticosterone levels (Sternberg, 2006). Furthermore, LPS administration causes a temporary reduction in feed intake (Star et al., 2008a), and, as for heat stress, this might have influence on physiological and metabolic processes, reflected by changes in endocrine and oxidative stress parameters. Although the effects of LPS administration on physiological responses such as fever, body temperature, and BW gain has received considerable attention, the effect on endocrine and oxidative stress responses is hardly described.

Understanding the interaction between heat stress and microbial challenge is important because 1) both are common in poultry farming and can occur together and 2) the two

stressors represent two physiological drives that utilize some common effectors but induce opposite responses (Blatteis, 2000). Studies on the response to infection (as induced by LPS) during heat exposure are lacking in poultry.

In the present study, effects of single or combined environmental stressors on endocrine and oxidative stress responses were investigated in 4 layer lines. Exposure to a high temperature (climatic stress) for 23 d and single administration of the model antigen LPS (hygienic stress) were used as environmental stressors. According to Blatteis (2000), we hypothesized that chickens are able to cope with single environmental stressors, but that problems in coping ability occur when chickens are exposed to combined environmental stressors. To evaluate the coping ability of the different layer lines the effects of climatic and hygienic stress on performance and physiological responses were investigated. The current paper will focus on effects of single or combined exposure to high temperature and LPS administration on physiological (endocrine and oxidative stress) responses in 4 layer lines. Endocrine and oxidative stress responses were studied in the form of levels of corticosterone, T3, glucose, UA, and TBARS. In an accompanying paper (Star et al., 2008a), effects of exposure to climatic and hygienic stress on performance of these layer lines is reported.

MATERIALS AND METHODS

Chickens, housing, and feed

Four purebred layer lines from Hendrix Genetics (Boxmeer, the Netherlands) were used: 3 White Leghorn lines (WA, WB, and WF) and 1 Rhode Island Red line (B1). These lines were characterized for low or high survival rate and low or high natural humoral immune competence as determined in a previous study (Star et al., 2007a). Line WA and WF were characterized for high survival rate and, respectively, a low and high natural humoral immune competence, whereas line WB and B1 were characterized for low survival rate and, respectively, a low and high natural humoral immune competence.

At 22 wk of age, 80 hens per line (320 in total) were transported from a housing facility of Hendrix Genetics to 2 identical climate respiration chambers of Wageningen University. In each climate chamber 40 hens per line were individually housed in battery cages (45 cm height × 40 cm depth × 24 cm width). The lines were randomly divided over the cages. Hens were fed a standard commercial phase 1 diet (159 g/kg crude protein, 39 g/kg crude fiber, and 11.8 MJ of ME/kg). Hens had free access to feed and water. At 22 wk of age, hens were kept at a 13L:11D light scheme. In each of the following 2 wk, the light period

was increased by 1 h. At the start of the experimental period (at 24 wk of age), hens were kept at a 15L:9D light scheme until the end of the experimental period (27 wk of age). Hens received routine vaccinations to Marek's disease (d 1), Newcastle disease (wk 2, 6, 12, 15), infectious bronchitis (d 1, wk 2, 10, 12, 15), infectious bursal disease (wk 3, 15), fowl pox (wk 15), and avian encephalomyelitis (wk 15). Beak trimming was not performed.

EXPERIMENTAL DESIGN

After an adaptation period of 12 d (temperature maintained at 21ºC), hens in the first climate chamber were exposed to acute heat stress. Within approximately 1 h, the temperature in this chamber increased from 21ºC to 32ºC, and was maintained at 32ºC during the following 23 d. In the second chamber (control), the temperature was maintained at 21ºC. At d 1 after the start of heat stress, half of the hens of the heat treatment and half of the control hens were i.v. injected with 1 mg/kg of BW of *Escherichia coli* LPS (serotype O55:B5, Sigma Chemical Co., St. Louis, MO). The remaining hens received a placebo treatment of PBS. An overview of the experimental design is given in Table 6.1. The Institutional Animal Care and Use Committee of Wageningen University approved the experimental protocol.

TABLE 6.1. Experimental design[1]

Treatment group	Temperature	LPS[2]	# animals	# animals per line
21ºC + PBS	21ºC	-	80	20
21ºC + LPS	21ºC	+	80	20
32ºC + PBS	32ºC	-	80	20
32ºC + LPS	32ºC	+	80	20

[1] The experiment consisted of 4 treatment groups, which were exposed to a temperature of 21 or 32ºC during 23 d, and were i.v. injected with *Escherichia coli* lipopolysaccharide (LPS) at d 1 after the start of the heat stress period. Within each treatment group, 4 genetically different purebred layer lines, characterized by natural humoral immune competence and survival rate, were equally represented. Line B1 was characterized by a low survival rate and high natural humoral immune competence, line WA was characterized by a high survival rate and low natural humoral immune competence, line WB was characterized by a low survival rate and low natural humoral immune competence, and line WF was characterized by a high survival rate and high natural humoral immune competence.

[2] + = hens injected with 1 mg/kg of BW of LPS; - = hens injected with PBS.

Endocrine and oxidative blood parameters

Blood samples were collected from the wing vein of all 320 individual hens at d -5, 2, 8, 15, and 22 after the start of heat stress. After sampling, blood was centrifuged and plasma was stored at -20ºC until further processing.

Corticosterone. Corticosterone concentrations in plasma samples collected from each chicken were quantified using a sensitive and highly specific commercial radioimmunoassay (RIA) kit (IDS Inc., Boldon, UK). Before assay, plasma samples were heated at 80ºC for 10 min to inactivate corticosterone-binding proteins. Corticosterone concentrations were expressed in nanograms per milliliter of plasma.

3,5,3'-Triiodothyronine. Plasma concentration of T3 was measured by RIA according to Darras et al. (1991). Measurements were performed using a commercial available T3 antiserum (ByK-Sangtec Diagnostica GmbH, Dietzenbach, Germany) in combination with a specific tracer (Amersham International, Slough, UK). Concentrations of T3 were expressed in nanograms per milliliter of plasma.

Glucose and uric acid. Plasma concentrations of glucose and UA were measured by commercial colorimetric diagnostic kits (glucose: IL Test kit, No. 182508-00; UA: IL Test kit, No. 181685-00), using the Monarch 2000 Chemistry System Model 760 (Monarch Chemistry System, Instrumentation Laboratories, Zaventem, Belgium). Glucose and UA concentrations were expressed in milligrams per deciliter of plasma.

Thiobarbituric acid reacting substances. Lipid peroxidation was measured by spectrophotometric determination of TBARS with a modified method described by Lin et al. (2004a,b). Levels of TBARS were expressed as nanomoles of malondialdehyde per milliliter of plasma.

Statistical analysis

Differences in levels of corticosterone, T3, glucose, UA, or TBARS were analyzed by a 4-way ANOVA for the effect of line, temperature, LPS administration, time, and their interactions by repeated measurement procedure using a 'hen nested within line, temperature, and LPS administration' option. Corticosterone and T3 were measured at d -5, 2, and 8 after the start of heat stress, whereas glucose, UA, and TBARS were measured at d -5, 2, 8, 15, and 22 after the start of heat stress.

Mean differences among lines and treatments were tested with Bonferroni's test. The PROC MIXED procedure of SAS was used for statistical analysis (SAS Institute, 2004). Effects were considered significant at $P < 0.05$.

RESULTS

CORTICOSTERONE

Main effects of exposure to heat, LPS administration, or combined exposure to heat and LPS are shown in Figure 6.1 and will be described first, whereas more detailed results per line are given in Table 6.2 and will be described after the main effects.

Corticosterone levels were increased by heat stress ($P < 0.0001$). A significant heat × time interaction ($P < 0.0001$) was found, in which hens exposed to heat stress (32ºC + PBS and 32ºC + LPS) had higher corticosterone levels than hens housed at the control temperature (21ºC + PBS and 21ºC + LPS) at d 2 ($P < 0.01$) and d 8 ($P < 0.0001$) after the start of heat stress. Administration of LPS had no effect on corticosterone levels.

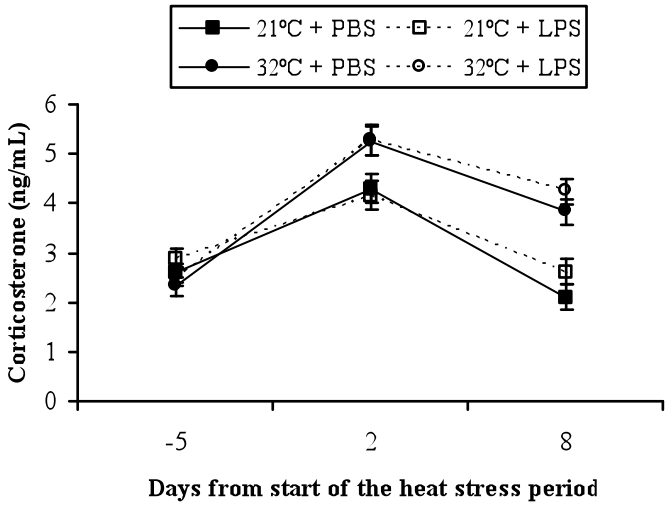

FIGURE 6.1. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on plasma corticosterone levels (ng/mL) of laying hens at d -5, 2, and 8 after the start of heat stress. Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1 after the start of the heat stress period.

TABLE 6.2. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on levels of corticosterone and 3,5,3'-triiodothyronine (T3) in 4 genetically different purebred layer lines

Line	Treatment	Corticosterone (ng/mL)[1]	T3 (ng/mL)[1]
B1	21ºC + PBS	2.91	0.27
	21ºC + LPS	3.84	0.29
	32ºC + PBS	2.91	0.23
	32ºC + LPS	3.61	0.23
WA	21ºC + PBS	2.75	0.30
	21ºC + LPS	2.16	0.34
	32ºC + PBS	3.31	0.29
	32ºC + LPS	3.44	0.35
WB	21ºC + PBS	3.66[b]	0.28[b]
	21ºC + LPS	4.60[ab]	0.37[ab]
	32ºC + PBS	5.68[a]	0.32[ab]
	32ºC + LPS	5.19[ab]	0.43[a]
WF	21ºC + PBS	2.66	0.20
	21ºC + LPS	2.28	0.22
	32ºC + PBS	3.32	0.20
	32ºC + LPS	3.82	0.27
	SEM	0.33	0.02

[a-b] Means with different superscript within treatment group of each line differ significantly ($P < 0.05$).

[1] Differences in corticosterone or T3 levels were analyzed by a 4-way ANOVA for the effect of line, temperature, LPS administration, time, and their interactions by a repeated measurement procedure using a 'hen nested within line, temperature, and LPS administration' option. Values are least squares means of corticosterone or T3 levels measured at d -5, 2, and 8 after the start of heat stress.

The 4 layer lines differed in corticosterone level ($P < 0.0001$; Table 6.2). Line WB had at each sample day significantly higher corticosterone levels than line B1, WA, and WF. Furthermore, there was a line × heat interaction ($P < 0.05$), indicating that lines differed in corticosterone levels in response to heat stress.

3,5,3'-TRIIODOTHYRONINE

Main effects of exposure to heat, LPS administration, or combined exposure to heat and LPS are shown in Figure 6.2 and will be described first, whereas more detailed results per line are given in Table 6.2 and will be described after the main effects.

TABLE 6.2, CONTINUED. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on levels of corticosterone and 3,5,3'-triiodothyronine (T3) in 4 genetically different purebred layer lines

Line	Treatment	Corticosterone (ng/mL)[1]	T3 (ng/mL)[1]
Effects	Line (L)	***	***
		WB > B1, WF, WA	WB, WA > B1, WF
	Temperature (T)	***	NS
		32ºC > 21ºC	
	LPS administration (A)	NS	***
			LPS > PBS
	Time (Ti)	***	***
	L × T	*	*
	L × A	NS	NS
	L × Ti	NS	**
	T × A	NS	NS
	T × Ti	***	***
	A × Ti	NS	***
	L × T × A	NS	NS
	L × T × Ti	NS	NS
	L × A × Ti	NS	**
	T × A × Ti	NS	NS
	L × T × A × Ti	NS	NS

*** P < 0.0001; ** P < 0.01; * P < 0.05; NS = not significant

There was a significant heat × time interaction (P < 0.0001). Before the start of heat stress (at d -5), hens in the chamber prepared for heat stress had higher T3 levels than hens in the chamber prepared for control temperature (P < 0.01). A more important difference caused by heat stress was found at d 2 after the start of heat stress, in which hens exposed to heat stress (32ºC + PBS and 32ºC + LPS) had lower T3 levels than hens exposed to the control temperature (21ºC + PBS and 21ºC + LPS; P < 0.0001). Furthermore, there was a significant LPS × time interaction (P < 0.0001); LPS administration (21ºC + LPS and 32ºC + LPS) increased T3 levels compared with PBS-treated hens (21ºC + PBS and 32ºC + PBS) at d 2 (P < 0.0001) and d 8 (P < 0.01) after the start of heat stress.

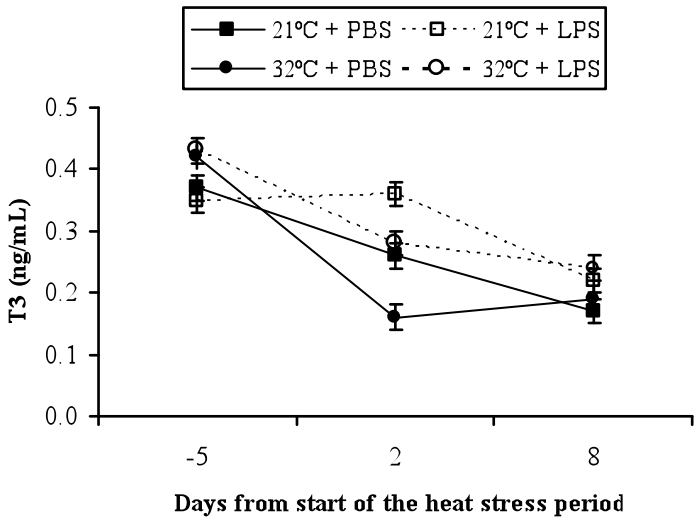

FIGURE 6.2. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on plasma 3,5,3'-triiodothyronine levels (T3; ng/mL) of laying hens at d -5, 2, and 8 after the start of heat stress. Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1 after the start of the heat stress period.

The 4 layer lines differed in T3 level ($P < 0.0001$; Table 6.2), in which line WA and WB had higher T3 levels than line B1 and WF. Besides, lines differed in T3 levels in response to heat stress (line × heat interaction; $P < 0.05$); T3 levels of line B1 were decreased by heat stress, T3 levels of line WB were increased, and T3 levels of line WA and WF were comparable to the control groups (21ºC + PBS) of these lines. Furthermore, T3 levels during the complete observation period were affected by a line × LPS × time interaction ($P < 0.01$; Table 6.2).

GLUCOSE

Main effects of exposure to heat, LPS administration, or combined exposure to heat and LPS are shown in Figure 6.3 and will be described first, whereas more detailed results per line are given in Table 6.3 and will be described after the main effects.

There was a significant heat × time interaction ($P < 0.0001$), in which hens exposed to heat stress (32ºC + PBS and 32ºC + LPS) had higher glucose levels than hens exposed to the control temperature (21ºC + PBS and 21ºC + LPS) at d 22 ($P < 0.0001$) after the start of heat stress. Furthermore, there was a significant LPS × time interaction ($P < 0.01$), in which

glucose levels in LPS-administered hens (21ºC + LPS and 32ºC + LPS) was lower at d 2 and 8 and higher at d 15 and 22 compared with PBS-treated hens (21ºC + PBS and 32ºC + PBS).

The 4 layer lines differed in glucose level ($P < 0.01$; Table 6.3), in which line WB and WF had higher glucose levels than line WA (line B1 was in-between). There was also a line × time interaction ($P < 0.05$), which suggests that lines had a different response pattern; line B1 and WB had an increased glucose level between d 2 and 8 after the start of heat stress, whereas line WA and WF had a decreased glucose level between d 2 and 8.

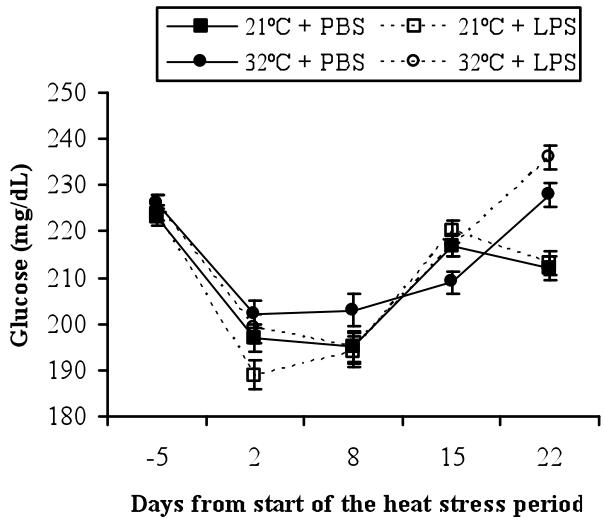

FIGURE 6.3. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on plasma glucose levels (mg/dL) of laying hens at d -5, 2, 8, 15, and 22 after the start of heat stress. Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1 after the start of the heat stress period.

URIC ACID

Main effects of exposure to heat, LPS administration, or combined exposure to heat and LPS are shown in Figure 6.4 and will be described first, whereas more detailed results per line are given in Table 6.3 and will be described after the main effects.

There was a significant heat × time interaction ($P < 0.0001$), in which hens exposed to heat stress (32ºC + PBS and 32ºC + LPS) had lower UA levels than hens exposed to the control temperature (21ºC + PBS and 21ºC + LPS) at d 8, 15 (both $P < 0.0001$), and 22 ($P <$

0.01) after the start of heat stress. Furthermore, there was a significant heat × LPS × time interaction ($P < 0.01$). At d 2 after the start of heat stress, hens exposed to the control temperature and administered with LPS (21ºC + LPS) had lower UA levels than hens exposed to the control temperature and treated with PBS (21ºC + PBS), whereas hens exposed to heat stress and administered with LPS (32ºC + LPS) or treated with PBS (32ºC + PBS) did not differ during the complete observation period.

The 4 layer lines differed in UA level ($P < 0.0001$; Table 6.3), in which line B1 and WB had higher UA levels than line WA and WF. There was also a line × time interaction ($P < 0.0001$), indicating that the lines had a different response pattern; lines WA, WB, and WF had an increase in UA levels from d 2 to d 15 after the start of heat stress whereafter the UA levels stabilized, whereas line B1 had an increase in UA level from d 2 to d 8 after the start of heat stress whereafter the UA levels stabilized.

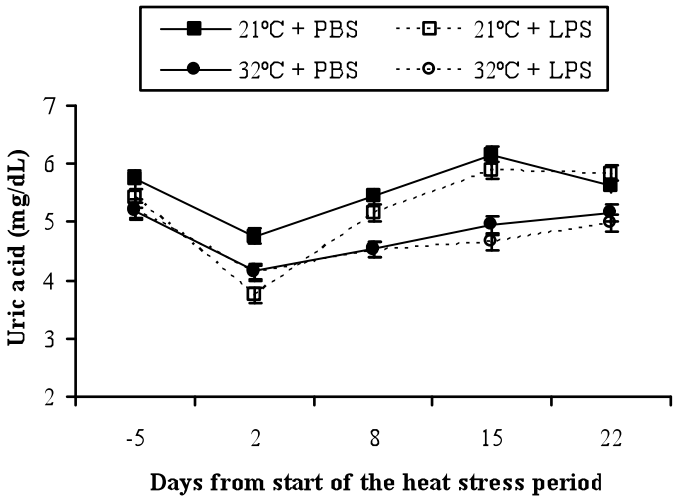

FIGURE 6.4. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on plasma uric acid levels (mg/dL) of laying hens at d -5, 2, 8, 15, and 22 after the start of heat stress. Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1 after the start of the heat stress period.

THIOBARBITURIC ACID REACTING SUBSTANCES

Main effects of exposure to heat, LPS administration, or combined exposure to heat and LPS are shown in Figure 6.5 and will be described first, whereas more detailed results per line are given in Table 6.3 and will be described after the main effects.

There was a significant heat × time interaction ($P < 0.01$). Before the start of heat stress (at d -5), hens in the chamber prepared for heat stress had higher levels of TBARS than hens in the chamber prepared for control temperature ($P < 0.01$). Furthermore, hens exposed to heat stress (32ºC + PBS and 32ºC + LPS) had higher levels of TBARS than hens exposed to the control temperature (21ºC + PBS and 21ºC + LPS; $P < 0.01$) at d 8 after the start of heat stress. Neither LPS administration nor line affected levels of TBARS (Table 6.3).

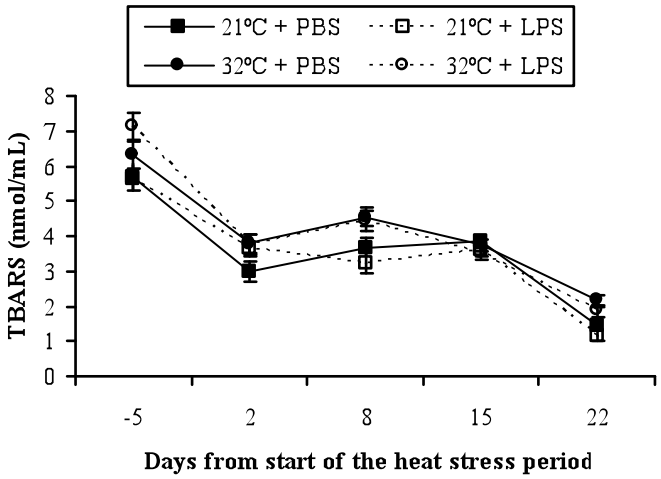

FIGURE 6.5. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on plasma levels of thiobarbituric acid reacting substances (TBARS; nmol/mL) of laying hens at d -5, 2, 8, 15, and 22 after the start of heat stress. Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1 after the start of the heat stress period.

DISCUSSION

In the present study, heat stress and LPS administration acted like 2 independent stressors, illustrated by the lack of interactions between heat stress and LPS administration (except for UA). This is not in accordance with our hypothesis. We had expected that chickens were able to cope with single environmental stressors, but that problems in coping ability

TABLE 6.3. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on levels of glucose, uric acid, and thiobarbituric acid reacting substances (TBARS) in 4 genetically different purebred layer lines

Line	Treatment	Glucose (mg/dL)[1]	Uric acid (mg/dL)[1]	TBARS (nmol/mL)[1]
B1	21ºC + PBS	210.7	6.15	3.35
	21ºC + LPS	207.4	5.23	3.11
	32ºC + PBS	211.5	5.05	3.78
	32ºC + LPS	210.9	5.06	3.98
WA	21ºC + PBS	205.4	5.13	3.73
	21ºC + LPS	202.5	5.01	3.63
	32ºC + PBS	210.6	4.50	3.97
	32ºC + LPS	211.9	4.51	3.99
WB	21ºC + PBS	207.1	5.73	3.80
	21ºC + LPS	212.7	5.61	3.41
	32ºC + PBS	214.8	5.16	4.31
	32ºC + LPS	217.2	4.95	4.18
WF	21ºC + PBS	211.3	5.17	3.27
	21ºC + LPS	209.7	4.99	3.68
	32ºC + PBS	218.0	4.49	4.34
	32ºC + LPS	218.2	4.32	4.43
	SEM	2.38	0.19	0.27

[1] Differences in levels of glucose, uric acid, or TBARS were analyzed by a 4-way ANOVA for the effect of line, temperature, LPS administration, time, and their interactions by a repeated measurement procedure using a 'hen nested within line, temperature, and LPS administration' option. Values are least squares means of levels of glucose, uric acid, or TBARS measured at d -5, 2, 8, 15, and 22 after the start of heat stress.

would occur when chickens were exposed to combined environmental stressors.

The 4 purebred lines used in the present study were characterized in a previous study (Star et al., 2007a) for high or low natural immune competence and high or low survival rate. Lines B1 and WF were selected for high natural immune competence, whereas lines WA and WB were selected for low natural immune competence. It was expected that endocrine and, to a lesser extent, oxidative stress responses would be related to natural immune competence, because immune responses are affected by the release of hormones by the hypothalamic-pituitary-adrenal axis (Mashaly et al., 1998; Sternberg, 2006). However,

TABLE 6.3, CONTINUED. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on levels of glucose, uric acid level, and thiobarbituric acid reacting substances (TBARS) in 4 genetically different purebred layer lines

Line	Treatment	Glucose (mg/dL)[1]	Uric acid (mg/dL)[1]	TBARS (nmol/mL)[1]
Effects	Line (L)	**	***	NS
		WF, WB ≥ B1 ≥ WA	B1, WB > WA, WF	
	Temperature (T)	***	***	***
		32ºC > 21ºC	21ºC > 32ºC	32ºC > 21ºC
	LPS administration (A)	NS	*	NS
			PBS > LPS	
	Time (Ti)	***	***	***
	L × T	NS	NS	NS
	L × A	NS	NS	NS
	L × Ti	*	***	NS
	T × A	NS	NS	NS
	T × Ti	***	***	**
	A × Ti	**	*	NS
	L × T × A	NS	NS	NS
	L × T × Ti	NS	NS	NS
	L × A × Ti	NS	NS	NS
	T × A × Ti	NS	**	NS
	L × T × A × Ti	NS	NS	NS

*** P < 0.0001; ** P < 0.01; * P < 0.05; NS = not significant.

in the present study, natural humoral immune competence seemed not related with endocrine and oxidative stress responses to different stressors.

In the present study, the 4 layer lines differed in response patterns and in response levels. The endocrine system of line WB was most sensitive for environmental changes, based on the observed differences in levels of corticosterone and T3 between the treatment groups within this line. In addition, performance parameters (Star et al., 2008a) and immune competence (Star et al., 2007b) of line WB were less affected by environmental changes. Performance parameters and immune competence of line B1 were most affected by environmental changes, and in addition, line B1 showed a low sensitive endocrine response

to environmental stressors. Like Wingfield and Kitaysky (2002), who suggested that glucocorticoids function as anti-stress hormones, we speculate that line B1 and WB have different mechanisms to cope with stress, which is probably based on the endocrine responsiveness, and especially on the glucocorticoid hormone corticosterone, to stressors. Corticosterone, as a response upon stress, may be beneficial in the interpretation that sensitivity to this hormone enables line WB to maintain performance together with low sensitivity of immune competence, whereas the stress response of line B1 is to a lesser extent under control of corticosterone resulting in sensitivity in immune competence and inability to maintain performance.

In the present study, the level of T3, as a thermoregulatory hormone, was temporarily decreased at d 2 after the start of heat stress. A decreased T3 level after heat exposure was also found in other studies (Geraert et al., 1996; Lin et al., 2000,2006a; Maak et al., 2003; Uni et al., 2001). In the present study, heat stress increased levels of corticosterone and glucose. The metabolic effect of elevated corticosterone levels is to provide glucose by gluconeogenesis (Davis et al., 2000). Furthermore, heat stress decreased plasma UA levels, as also found by Lin et al. (2000), whereas other studies (Geraert et al., 1996; Koelkebeck and Odom, 1995; Lin et al., 2006a) found no effect of heat stress on UA levels. The decrease in UA levels might be related to the weight loss of the chickens (Star et al., 2008a), because there is a positive correlation between plasma UA levels and BW loss (Tsahar et al., 2006). The reduction in feed intake (Star et al., 2008a) probably caused the weight loss and, related to this, the changes in UA level. Interestingly, mechanisms underlying changes in glucose and UA level are probably different between reduced feed intake due to heat stress or due to forced feed restriction. Forced feed restriction caused a decrease in glucose level (Nijdam et al., 2005) and increase in UA level (Buyse et al., 2002). The decrease in glucose level due to forced feed restriction is caused by a reduced glucose utilization and overall metabolism together with a shift from glucose to free fatty acid use (Buyse et al., 2002; Swennen et al., 2005). The increase in glucose level as a consequence of reduced feed intake due to heat stress is probably caused by a slight increase in gluconeogenesis linked with initial increased corticosterone levels, which could explain the opposite changes in glucose levels in both situations of reduced feed intake. The increase in UA level due to forced feed restriction is caused by an enhanced gluconeogenesis at the expense of body proteins (Buyse et al., 2002). The decrease in UA level as a consequence of reduced feed intake due to heat stress is probably caused by a decreased metabolic level. This could induce a protein-sparing effect (by down-regulation of protein turnover) as well as glucose-sparing by increased utilization of fat reserves.

Therefore, this weight loss due to heat exposure could result in decreased UA levels. Furthermore, the decreased UA level and enhanced level of TBARS suggests an increased oxidative stress caused by heat stress, indicating a disturbance in the balance between the oxidation and antioxidant defense system. Although there was no replication of the heat stress environment (possible confounded results), the findings of endocrine and oxidative stress responses, as well as the findings described in the accompanying paper (Star et al., 2008a), are in accordance with other studies.

The effect of microbial challenges, mimicked by LPS, on endocrine and oxidative stress responses of adult laying hens is not documented. Administration of LPS caused an increase in T3 levels, which is probably related to the drop in egg production as described in Star et al. (2008a). It has been found that T3 is associated with the reproductive state of the chicken. When there is a (fast) drop in egg production, for example during molt (Davis et al., 2000; Hoshino et al., 1988), plasma T3 levels will increase. Levels of UA were decreased by LPS administration, probably due to the reduction in feed intake, and similar as explained for the decrease of UA during heat stress. The reduction in feed intake by LPS administration, however, had no detectable effect on levels of corticosterone and TBARS. Lipopolysaccharide is an acute inflammatory stimulus, and LPS administration induces a rapid, short-lived increase in plasma corticosterone levels (Stenzel-Poore et al., 1993). In a study by Kluger et al. (1997), male rats were exposed to heat stress and were administered with LPS 24 h later. Corticosterone levels were measured, and injection of LPS led to a marked rise in plasma corticosterone at 4 h, but not at 24 h post-injection. In the present study, blood samples were taken 1 d after LPS administration. The reaction of corticosterone on LPS treatment was probably more acute and took place within 1 d after treatment, which might explain why no effect of LPS on corticosterone was found. The effect of LPS administration on TBARS is, to our knowledge, not studied before. Lin et al. (2004a,b) found that levels of TBARS were elevated for chronic and acute stress effects after corticosterone administration. In the present study, however, no effect of LPS administration on level of TBARS was found.

Under the given circumstances (temperature of 21 or 32ºC with or without 1 mg/kg of BW of LPS), the results of this study indicate that, based on endocrine and oxidative stress responses, hens were able to cope with single or combined heat stress and LPS administration. There were no interactions between heat stress and LPS administration, but both stressors caused a reduction in feed intake, and changes in plasma levels of the endocrine and oxidative stress parameters are partly related with the reduction in feed intake. Although line B1 showed the largest reduction in feed intake, it seems that line WB

reacted in the most sensitive way to environmental changes. Neither natural humoral immune competence nor survival rate, on which bases the lines were characterized, was indicative for the stress response to different stressors. The present data suggest that heat stress and LPS administration acted like two independent stressors and that the 4 layer lines differed in response patterns and response levels.

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CHAPTER 7

Effect of early-life thermal conditioning and immune challenge on thermo tolerance and humoral immune competence in adult laying hens

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ABSTRACT

1. Effects of early-life experience with climatic (heat) and hygienic [lipopolysaccharide (LPS)] stress on adaptability to the same stressors in later-life were studied in laying hens.

2. Chicks were exposed to 37ºC for 24 h at d 5 of age (n = 12), or were i.v. administered once with 1 mg/kg of BW of LPS at 6 wk of age (n = 12), or were exposed to both stressors (n = 12), while a control group was reared under standard conditions receiving a placebo treatment of PBS (n = 36). At 24 wk of age, hens treated in early-life were re-exposed to a similar stressor. Early-life control hens were exposed to heat stress (n = 12), i.v. administered with LPS (n = 12), or exposed to both stressors (n = 12).

3. No effect of early-life heat exposure on performance, immune, and endocrine parameters was found.

4. Treatment × Time interactions were found for level of antibody (Ab) binding to LPS and KLH after LPS administration, indicating that hens with early-life LPS experience differed in response level (Ab binding to LPS) and response pattern (Ab binding to LPS and KLH) compared to hens administered with LPS only at adult age.

5. Levels of Ab binding to LPS and KLH were comparable between hens exposed to combined heat and LPS and hens only exposed to LPS, indicating that heat stress had no additive effect.

6. Our data suggest that early-life heat stress exposure did not affect adaptability of laying hens to heat stress in later-life. However, early-life LPS exposure and combined early-life exposure to heat and LPS affected kinetics and magnitude of Ab levels binding to LPS and KLH, indicating that early-life LPS exposure can enhance the status of immune reactivity or induce a higher sensitivity to LPS.

Key words: environment, immunology, laying hen, physiology, temperature

Abbreviation key: Ab = antibody, APW = alternative complement pathway, BW = body weight, CPW = classical complement pathway, IBD = infectious bursal disease, KLH = keyhole limpet haemocyanin, LPS = lipopolysaccharide, MBL = mannan-binding lectin, NCD = Newcastle disease, PBS = phosphate buffered saline

INTRODUCTION

Animals can be conditioned to an expected change in the environment. For instance, broiler chickens can be physiologically modulated by thermal conditioning in early-life to improve heat stress tolerance in later-life (Arjona et al., 1990; De Basilio et al., 2001; Yahav and Hurwitz, 1996; Yahav and Plavnik, 1999). The main idea in the thermal conditioning process is to manipulate immature mechanisms of temperature regulation in early-life, enabling chickens to cope, within certain limits, with acute exposure to unexpected heat spells in later-life (Yahav and Hurwitz, 1996; Yahav and McMurtry, 2001). In broiler chickens, thermal conditioning to heat at an early age resulted in reduced weight gain during the first wk of life, followed by an accelerated growth (Yahav and Plavnik, 1999; Yahav and McMurtry, 2001), improved thermo tolerance, and reduced mortality when re-exposed to heat in later-life (Arjona et al., 1990; De Basilio et al., 2001; Yahav and Hurwitz, 1996; Yahav and Plavnik, 1999). Early thermal conditioning seems to be one of the most promising methods to improve the adaptability of broiler chickens to heat stress (Lin et al., 2006b).

Such a conditioning process may also be used to prepare animals to hygienic conditions. Gram-negative bacteria are ubiquitous in the environment of poultry and, in particular, inhalation of their endotoxin has been recognized as an important factor in the prevalence of respiratory diseases (Zucker et al., 2000). One of the major endotoxins in the poultry environment is lipopolysaccharide (LPS). The endotoxin LPS is often used as a model antigen to study the animal's susceptibility to (non-specific components of microbiological) pathogens and capability to react to microbial stressors (Ulevitch and Tobias, 1999). In this respect, it is noteworthy that chickens become refractory to repeated LPS administrations, accompanied by a minimal decreasing effect on performance characteristics, e.g., feed intake and growth (Korver et al., 1998; Parmentier et al., 2006). Repeated LPS administrations seem to stimulate adaptation in an as yet to be identified mechanism and, therefore, might improve the adaptability to this stressor.

Early-life experience with climatic conditions has been studied in broilers, but not in laying hens. Early-life experience with hygienic conditions has potential, but effects on basic adaptation parameters, like hen-day egg production and feed intake, has not been studied before in laying hens. Besides, little is known about the effects of early-life experience with hygienic conditions on development of (natural) humoral immunity. In the present study, we hypothesized on the basis of the mentioned literature as reviewed above, that early-life experiences with climatic and hygienic stress would improve the adaptability

of laying hens to these stressors. To test this hypothesis, laying hens were exposed to heat stress and/or LPS administration in early-life, and re-exposed to similar stressors as adult hens during early lay. These stressor have also been used in previous studies (Star et al., 2007b,2008a,b), where adult hens were exposed to single or combined climatic stress (heat) and hygienic stress (LPS) during early lay. In the present study, the focus will be on improvement of adaptability, e.g., the effect of early-life experience, rather than the difference between climatic and hygienic stress responses. To evaluate the improvement of adaptability, effects of climatic and hygienic stress on performance (e.g., feed intake, BW, hen-day egg production, egg weight, and clutch length), humoral immune competence, and endocrine responsiveness were investigated in hens with early-life experience to the stressors and hens only exposed to the stressors in later-life.

TABLE 7.1. Experimental design

	Temperature (ºC)[1]		LPS[2]		Birds (n)[3]
Treatment group	Early-life	Adult	Early-life	Adult	
LPS - early-life	32	21	+	+	11
LPS - adult	32	21	-	+	12
Heat - early-life	37	32	-	-	12
Heat - adult	32	32	-	-	12
Heat + LPS - early-life	37	32	+	+	12
Heat + LPS - adult	32	32	-	+	12

[1] Temperature treatment at d 5 of age for 24 h (Early-life), and at 24 wk of age for 22 d (Adult).

[2] Administration of *Escherichia coli* lipopolysaccharide (LPS) (i.v. 1 mg/kg of BW) at 6 wk (Early-life) and 24 wk of age (Adult). + = hens injected with LPS; - = hens injected with PBS.

[3] Number of birds at 24 wk of age at the start of the experimental period.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

In this study, 72 purebred layer chicks (Rhode Island Red; line B1) were used. An overview of the experimental design is given in Table 7.1. At day of hatch, chicks were obtained from a hatchery (Hendrix Genetics, Boxmeer, the Netherlands), and placed in a climate respiration chamber (control chamber) at Wageningen University. The next day, 24 randomly chosen chicks were housed in 2 smaller climate chambers (12 chicks per

chamber) for 1 wk (d 1 to 7 after hatch). At d 5 after hatch, chicks in the small climate respiration chambers were exposed to heat stress. Within approximately 1 h, temperature in these 2 chambers increased from 32 to 37ºC, and was maintained at 37ºC during the following 24 h. In the control chamber, temperature was maintained at 32ºC. The age of 5 d and a temperature challenge of 37ºC were chosen according to thermal conditioning studies done in broilers (De Basilio *et al.*, 2001; Yahav and Hurwitz, 1996; Yahav and Plavnik, 1999).

At 6 wk of age, 24 chicks (12 chicks were also exposed to heat at d 5 of age) were i.v. administered with 1 mg/kg of BW of *Escherichia coli* LPS (serotype O55:B5, Sigma Chemical Co., St. Louis, MO). The remaining hens received a placebo treatment of PBS. The age of 6 wk was chosen because it was expected that the immune system of the chick was not fully mature yet, but that the influence of maternally derived immunity will be low at that age.

At 22 wk of age, 71 hens were moved to 2 identical climate respiration chambers (1 hen was removed from the experiment because of egg production problems), where they were individually housed in battery cages (45 cm height × 40 cm depth × 24 cm width). After an adaptation period of 12 d (temperature maintained at 21ºC), laying hens treated in early-life were re-exposed to a similar stressor (exposed to heat stress, i.v. administered once with 1 mg/kg of BW of LPS). Early-life control hens were exposed to heat stress (n = 12), i.v. administered with LPS (n = 12), or exposed to both stressors (n = 12).

Hens in the first climate chamber were exposed to heat. Within approximately 1 h, temperature in this chamber was increased from 21 to 32ºC, and was maintained at 32ºC during the following 22 d. In the second chamber, temperature was maintained at 21ºC. At d 1 after the onset of heat stress, hens were i.v. administered with 1 mg/kg of BW of *Escherichia coli* LPS. The remaining hens received a placebo treatment of PBS. After 22 d, temperature in the heat stress chamber was decreased to 21ºC until the end of the experiment 1 wk later. Each performance, immune, and endocrine parameter was established in each of the individual hens only at adult age.

The Institutional Animal Care and Use Committee of Wageningen University approved the experimental protocol.

HOUSING AND FEED

At the day of hatch, chicks were placed in a climate respiration chamber where temperature was set at 34ºC, and gradually reduced by 0.5ºC until 31ºC at the end of the first wk, except for the chicks housed in the small climate chambers that were exposed to heat stress at d 5

after hatch. Light was provided for 24 h at the first day and for 23 h during d 2 to 7. Hereafter, all chicks were reared under comparable conditions. The chicks were housed in cages, where temperature was reduced by 1ºC every 3 d until 21ºC was reached at d 36. The light period was reduced to 19 h after the first wk, and hereafter, until 16 wk of age, the light period was gradually reduced by 1 h every fortnight, and from 16 until 18 wk of age by 1 h weekly until hens were kept under a 9L:15D light scheme. Hereafter, the light period was increased by 1 h per week until hens were kept under a 15L:9D light scheme. During the experimental period (from 24 to 28 wk of age) hens were kept under a 15L:9D light scheme.

Chicks were fed a rearing phase 1 diet (202 g/kg crude protein, 45 g/kg crude fibre, and 11.1 MJ of ME/kg) until the age of 13 wk and a rearing phase 2 diet (176 g/kg crude protein, 46 g/kg crude fibre, and 12.1 MJ of ME/kg) until 16 wk of age. Hereafter, hens were fed a standard commercial laying phase 1 diet (155 g/kg crude protein, 34 g/kg crude fibre, and 11.8 MJ of ME/kg).

The hens received routine vaccinations against Marek's disease (d 1), Newcastle disease (NCD, d 10, 31, 103), infectious bronchitis (d 1, 76, 103), infectious bursal disease (IBD, d 21), fowl pox (d 56), infectious laryngotracheitis (d 85), and avian encephalomyelitis (d 94). Beak trimming was not performed.

Performance

During the adaptation and experimental period in the climate respiration chambers, feed and water were available ad libitum, where feed intake was recorded daily. Body weight was measured at d -5, 2, 8, 16, 22, and 29 after the start of heat stress at 24 wk of age. Body weight development in time was calculated in relation to BW measured at d 5 prior to the start of heat stress. Egg number was recorded daily during the adaptation and experimental period. Clutch length, i.e., mean number of eggs per clutch, was recorded during the experimental period. Egg weight was recorded weekly at d -3, 4, 11, 18, and 25 after the start of heat stress. Mortality was registered.

Immune and endocrine parameters

Blood samples for plasma and serum were collected from the wing vein of all 71 hens at d -5, 2, 5, 8, 16, 22, and 29 after the start of heat stress. After sampling, blood was centrifuged and both plasma and serum were stored at -20ºC until further processing. Plasma samples were used to analyse antibodies (Ab) and corticosterone, and serum samples were used to analyse haemolytic complement activity and mannan-binding lectin (MBL) concentration.

HUMORAL IMMUNE RESPONSE TO LPS AND KLH. Ab levels binding to LPS and natural Ab levels binding to keyhole limpet haemocyanin (KLH) were determined in individual samples by an indirect ELISA procedure at d -5, 2, 5, 8, 16, 22, and 29 after the start of heat stress. Flat-bottomed 96-well medium-binding ELISA plates were coated with either 4 µg/ml of *Escherichia coli* LPS or 1 µg/ml of KLH (MP Biomedicals Inc., Aurora, OH). After subsequent washing, plates were incubated with plasma (diluted 1:40, 1:160, 1:640, and 1:2,560 for LPS and KLH). Binding of Ab to LPS and KLH antigen was visualized using a 1:20,000 diluted rabbit anti-chicken IgG_{H+L} labelled with peroxidase (RACh/IgG_{H+L}/PO; Nordic, Tilburg, the Netherlands). After washing, substrate [tetramethylbenzidine (TMB) and 0.05% H_2O_2] was added, and 10 min later, the reaction was stopped with 2.5 *N* H_2SO_4. Extinctions were measured with a microplate reader (Multiskan, Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Levels (titers) were expressed as log2 values of the dilutions that gave an extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of a standard positive plasma present on each flat-bottomed ELISA plate.

HUMORAL IMMUNE RESPONSE TO NCD AND IBD VACCINES. Plasma samples collected at d -5, 2, 5, 8, 16, 22, and 29 after the start of heat stress were tested for the levels of Ab binding to the vaccine NCD (ND Clone 30, Intervet International BV, Boxmeer, the Netherlands) and IBD (Gumboro D78, Intervet International BV, Boxmeer, the Netherlands) using an indirect ELISA as described above. Plates were coated with 100 µl/well of a 1:50 solution of NCD dissolved in carbonate buffer or a 1:50 solution of IBD dissolved in carbonate buffer. Plasma samples were diluted 1:80, 1:320, 1:1,280, 1:5,120 for NCD and IBD.

HAEMOLYTIC COMPLEMENT ASSAY. Activity of the classical complement pathway (CPW) and alternative complement pathway (APW) were determined in individual serum samples collected at d -5, 2, 5, 8, 16, 22, and 29 after the start of heat stress. The haemolytic complement assay was performed according to the method described by Demey *et al.* (1993). Briefly, appropriate buffers were prepared. The buffer solution for CPW was prepared by adding $MgCl_2$ (1 mmol/l) and $CaCl_2$ (0.15 mmol/l) to Veronal-buffered saline. The buffer solution for APW was prepared by adding $MgCl_2$ (5 mmol/l) and ethylene glycol tetraacetate (16 mmol/l) to Veronal-buffered saline.

The assay was performed in flat-bottomed 96-well microtiter plates. Sera were diluted serially in the appropriate buffers and incubated with sensitized (ref. no. 72202,

Haemolysin, bioMérieux, Marcy l'Etoile, France) sheep erythrocytes or bovine erythrocytes prepared by standard methods and used as a 1% cell suspension to measure CPW or APW, respectively. During 1.5 h of incubation, plates were shaken every 30 min. Results (the amount of light scattering by erythrocytes upon lysis) were read in a microplate reader (Multiskan, Labsystems) at a wavelength of 690 nm. Readings were transformed by a log-log equation (Von Krogh, 1916), and the haemolytic titer was expressed as the titer that lyses 50% of the red blood cells (CH50 U/ml).

MBL complement assay. Activity of the mannan-binding lectin pathway of complement (MBL) was determined in individual serum samples by an indirect ELISA procedure at d -5, 2, 5, 8, 16, and 22 after the start of heat stress. The MBL assay was performed according to the method described by Norup and Juul-Madsen (2007). Briefly, flat-bottomed 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 5 µg/ml of anti-chicken MBL antibody (HYB 182-01, Statens Serum Institute, Copenhagen, Denmark). After washing, residual protein-binding sites were blocked by 200 µl 0.5% (v:v) Tween 20 in 10 m*M* Tris, 140 m*M* NaCl, for 33 min. The plates were then incubated with serum (diluted 1:610), for 2 h. After another washing step, plates were incubated with 1 µg/ml biotinylated mouse anti-chickenMBL (HYB 182-01), for 45 min, followed by a 30 min incubation with 1:13,000 diluted horseradish peroxidase (HRP)-conjugated-streptavidin (P0397, Dako, Glostrup, Denmark). Finally, the presence of HRP was detected by adding 100 µl of substrate (TMB). After 27 min at 26ºC, colour development was stopped with 100 µl 1 *M* H_2SO_4 and determined by reading the absorbance at 450 nm. MBL activity was expressed in microgram per millilitre serum.

Corticosterone. Corticosterone levels were determined in individual plasma samples by using a sensitive and highly specific commercial radioimmunoassay (RIA) kit (IDS, Inc., Boldon, UK) at d -5, 2, and 5 after the start of heat stress. Before performing the assay, plasma samples were heated at 80ºC for 10 min to inactivate corticosterone-binding proteins. Corticosterone concentrations were expressed in nanogram per millilitre plasma.

Statistical analysis

Differences between early-life and adult exposure to heat stress, LPS administration, or a combination of these stressors were established.

Differences in feed intake, hen-day egg production, and clutch length were analyzed by a 1-way ANOVA for the effect of treatment (Early-life vs. Adult). Feed intake and hen-day

egg production were, after primary analyses of the daily measurements, divided in 4 periods; P1 is the adaptation period (d -11 to 0), P2 is the experimental period where temperature and LPS administration were of influence (d 1 to 7), P3 is the experimental period where only temperature was of influence (d 8 to 22), and P4 is the recovery period after the end of heat stress. Statistical analyses were done for each period, but no statistical analyses were done among periods.

Differences in BW development, egg weight, corticosterone, and each of the immune parameters were analysed by a 2-way ANOVA for the effect of treatment (Early-life vs. Adult), time, and their interactions by a repeated measurement procedure using a 'hen nested within treatment' option.

Correlation between feed intake, hen-day egg production, and egg weight before the start of the experimental period were analysed by Pearson product-moment correlation. Data were not corrected for the effect of early-life treatment.

All analysis were carried out using SAS (SAS Institute, 2004). The PROC GLM procedure was used to analyse the 1-way-ANOVA, the PROC MIXED procedure was used to analyse the 2-way-ANOVA with a repeated measurement procedure, and the PROC CORR procedure was used to analyse correlations. Mean differences between treatments were tested with Bonferroni's test. Effects were considered significant at $P < 0.05$.

RESULTS

EARLY-LIFE EXPERIENCE WITH HEAT STRESS

No differences were found for BW development in time, feed intake, hen-day egg production, egg weight, clutch length, level of Ab binding to LPS, KLH, NCD, and IBD, and complement activity of CPW and MBL between hens with early-life heat stress experience and hens that were exposed to heat stress only at adult age (Table 7.2).

A difference was observed in complement activity of APW ($P < 0.05$; Table 7.2). This difference was, however, not caused by the actual heat stress. The heat stress treatments differed in APW activity already before the start of heat stress at 24 wk of age.

A difference was also observed in corticosterone levels ($P < 0.05$; Table 7.2), where corticosterone levels of hens exposed to heat stress only at adult age were higher than corticosterone levels of hens with early-life heat stress experience. This difference was probably not caused by the actual heat stress. Before the start of heat stress, corticosterone levels of hens exposed to heat stress only at adult age were already higher than corticosterone levels of hens with early-life heat stress experience.

TABLE 7.2. Effects of early-life exposure to heat stress on performance, immune, and endocrine parameters in later-life. Treatment Early-life are laying hens exposed to heat stress (37ºC) at d 5 of age and repeated exposed to heat stress (32ºC) at 24 wk of age for 22 d, whereas treatment Adult are laying hens only exposed to heat stress (32ºC) at 24 wk of age for 22 d

		Treatment		P-value		
Parameter		Early-life	Adult	Treatment	Time	Treatment × Time
Feed intake (g/d) [1]	P1	82.4	83.7	0.8412		
	P2	52.4	48.6	0.5247		
	P3	65.2	70.7	0.3196		
	P4	101.1	97.2	0.5289		
Egg production (%)[1]	P1	70.1	71.5	0.9141		
	P2	54.8	50.0	0.6532		
	P3	60.6	57.2	0.7698		
	P4	84.5	71.4	0.3011		
Clutch length		3.5	3.2	0.7016		
Body weight gain (g)[2]		-108	-148	0.3224	<0.0001	0.8416
Egg weight (g)		50.0	50.9	0.5011	<0.0001	0.3924
Complement	CPW	438	455	0.7536	0.0003	0.6424
(CH50-value)	APW	209	150	0.0233	0.0184	0.1904
(μg/ml)	MBL	17.6	20.6	0.3771	<0.0001	0.9165
Ab titer	LPS	3.0	2.8	0.5771	<0.0001	0.4185
	KLH	4.5	4.2	0.3262	<0.0001	0.1952
	NCD	7.1	6.4	0.2455	0.0047	0.7428
	IBD	2.0	2.0	0.9159	0.0012	0.9013
Corticosterone (ng/ml)		2.3	3.1	0.0101	0.0322	0.9592

[1] Data on feed intake and hen-day egg production were divided into 4 periods; P1 is the adaptation period (d -11 to 0), P2 is the first week of the experimental period (d 1 to 7), P3 is the second and third wk of the experimental period (d 8 to 22), and P4 is the recovery period after the end of heat stress (d 23 to 29).

[2] The BW development in time was calculated compared to BW at d 5 prior to the start of heat stress.

EARLY-LIFE EXPERIENCE WITH LPS ADMINISTRATION

Levels of Ab binding to LPS were increased after LPS administration. Hens with early-life LPS experience had a different response level and response pattern compared to hens administered with LPS only at adult age (Treatment × Time interaction, $P < 0.0001$; Table

7.3). Hens administered with LPS only at adult age showed a primary immune response with a peak in Ab level at d 7 after LPS administration. Hens with early-life LPS experience showed a secondary immune response with a peak in Ab level at d 4 after LPS administration (Figure 7.1A).

Levels of natural Ab binding to KLH were increased after LPS administration. Hens with early-life LPS experience had a different response pattern compared to hens administered with LPS only at adult age (Treatment × Time interaction, $P < 0.01$; Table 7.3). Hens administered with LPS only at adult age had a peak in natural Ab level at d 7 after LPS administration, whereas hens with early-life LPS experience had a peak in natural Ab level at d 4 after LPS administration (Figure 7.2A).

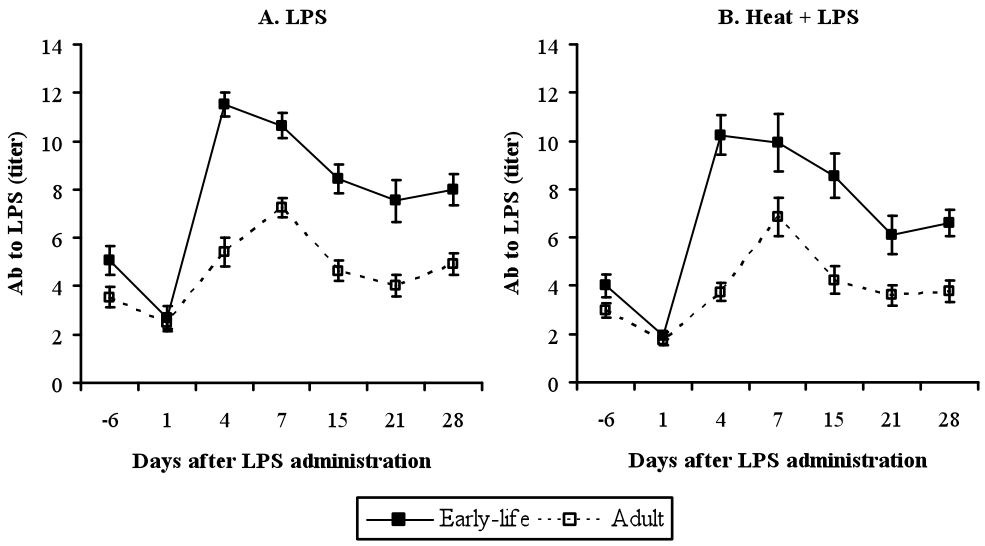

FIGURE 7.1. Effect of early-life exposure to [A] *Escherichia coli* lipopolysaccharide administration (LPS; i.v. 1 mg/kg of BW) and [B] combined heat stress and *Escherichia coli* lipopolysaccharide administration (heat + LPS) on level of antibody (Ab) binding to LPS in later-life. Treatment Early-life are laying hens administered with LPS at 6 wk of age and repeated administration of LPS at 24 wk of age, whereas treatment Adult are laying hens only administered with LPS at 24 wk of age.

TABLE 7.3. Effect of early-life exposure to *Escherichia coli* lipopolysaccharide (LPS) administration (i.v. 1 mg/kg of BW) on performance, immune, and endocrine parameters in later-life. Treatment Early-life are laying hens administered with LPS at 6 wk of age and repeated administration of LPS at 24 wk of age, whereas treatment Adult are laying hens only administered with LPS at 24 wk of age

		Treatment		P-value		
Parameter		Early-life	Adult	Treatment	Time	Treatment × Time
Feed intake (g/d)[1]	P1	91.1	81.6	0.2411		
	P2	77.3	70.7	0.4648		
	P3	100.9	97.5	0.6825		
	P4	110.9	96.7	0.0328		
Egg production (%)[1]	P1	70.5	50.9	0.2711		
	P2	34.3	27.0	0.5223		
	P3	90.7	77.8	0.2481		
	P4	95.7	95.2	0.8843		
Clutch length		8.1	7.4	0.6633		
Body weight gain (g)[2]		14	27	0.6517	0.0042	0.9389
Egg weight (g)		56.1	53.5	0.0482	<0.0001	0.3746
Complement	CPW	492	538	0.3414	<0.0001	0.6665
(CH50-value)	APW	192	220	0.1511	<0.0001	0.7842
(μg/ml)	MBL	17.8	19.0	0.5937	<0.0001	0.2993
Ab titer	LPS	7.1	4.6	0.0034	<0.0001	<0.0001
	KLH	5.8	6.0	0.7375	<0.0001	0.0012
	NCD	6.8	6.6	0.3880	0.5234	0.7158
	IBD	3.0	2.5	0.1329	<0.0001	0.2113
Corticosterone (ng/ml)		2.2	2.1	0.6606	0.0028	0.2132

[1] Data on feed intake and hen-day egg production were divided into 4 periods; P1 is the adaptation period (d -11 to 0), P2 is the first week of the experimental period (d 1 to 7), P3 is the second and third wk of the experimental period (d 8 to 22), and P4 is the recovery period after the end of heat stress (d 23 to 29).

[2] The BW development in time was calculated compared to BW at d 5 prior to the start of heat stress.

During P4, feed intake was higher for hens with early-life LPS experience compared to hens that were administered with LPS only at adult age ($P < 0.05$, Table 7.3). Hens with early-life LPS experience, however, had already a higher feed intake during P1. Between P1 and P4, feed intake increased by 21.7% in hens with early-life LPS experience and with

18.5% in hens that were administered with LPS only at adult age. A higher feed intake in hens with early-life LPS experience is probably related to the slightly higher hen-day egg production and higher egg weight ($P < 0.05$). Before the start of the experimental period, positive correlations were found between feed intake and hen-day egg production ($r = 0.64$, $P < 0.0001$), between feed intake and egg weight ($r = 0.31$, $P < 0.05$), and between hen-day egg production and egg weight ($r = 0.41$, $P < 0.01$).

No differences were found for BW development in time, hen-day egg production, clutch length, level of Ab binding to NCD and IBD, complement activity of CPW, APW, and MBL, and corticosterone level between hens with early-life LPS experience and hens that were administered with LPS only at adult age (Table 7.3). One laying hen with early-life LPS experience died within 1 d after repeated LPS administration at 24 wk of age.

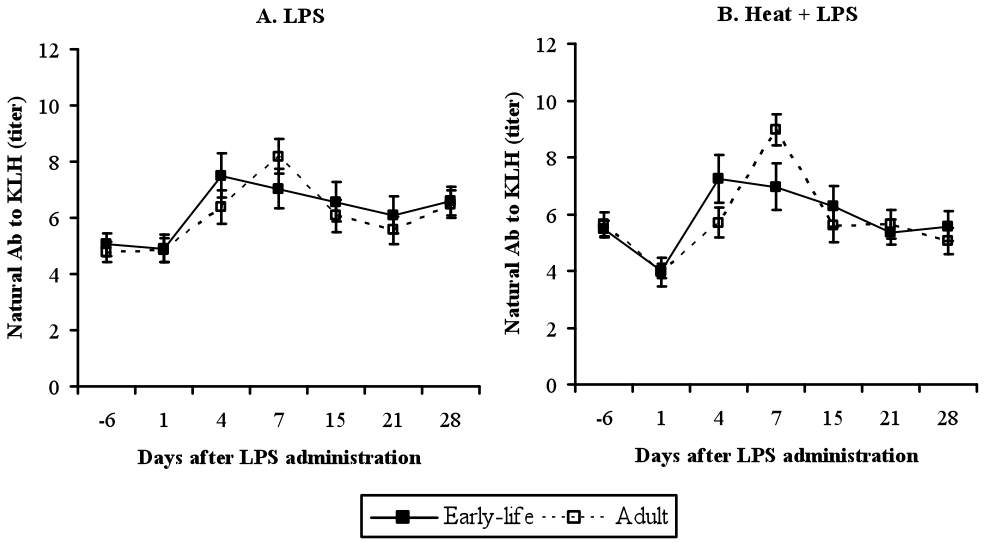

FIGURE 7.2. Effect of early-life experience to [A] *Escherichia coli* lipopolysaccharide administration (LPS; i.v. 1 mg/kg of BW) and [B] combined heat stress and *Escherichia coli* lipopolysaccharide administration (heat + LPS) on level of natural antibody (Ab) binding to KLH in later-life. Treatment Early-life are laying hens administered with LPS at 6 wk of age and repeated administration of LPS at 24 wk of age, whereas treatment Adult are laying hens only administered with LPS at 24 wk of age.

TABLE 7.4. Effect of early-life exposure to heat stress and *Escherichia coli* lipopolysaccharide (LPS) administration (i.v. 1 mg/kg of BW) on performance, immune, and endocrine parameters in later-life. Treatment Early-life are laying hens exposed to heat stress (37ºC) at d 5 of age and administered with LPS at 6 wk of age, and repeated exposed to heat stress (32ºC for 22 d) and administration of LPS at 24 wk of age, whereas treatment Adult are laying hens only exposed to heat stress (32ºC for 22 d) and administered with LPS at 24 wk of age

		Treatment		P-value		
Parameter		Early-life	Adult	Treatment	Time	Treatment × Time
Feed intake (g/d)[1]	P1	82.9	92.3	0.1914		
	P2	45.5	45.9	0.9389		
	P3	61.0	69.4	0.0464		
	P4	97.3	107.1	0.3103		
Egg production (%)[1]	P1	54.2	67.4	0.4064		
	P2	21.4	14.3	0.1977		
	P3	51.3	55.2	0.7972		
	P4	70.0	89.8	0.2198		
Clutch length		3.0	4.4	0.1189		
Body weight gain (g)[2]		-94	-77	0.6601	<0.0001	0.6979
Egg weight(g)		49.7	49.4	0.8010	<0.0001	0.2049
Complement	CPW	495	565	0.1565	<0.0001	0.8818
(CH50-value)	APW	187	223	0.1550	<0.0001	0.5886
(μg/ml)	MBL	19.9	19.9	0.9902	<0.0001	0.1176
Ab titer	LPS	6.0	2.9	<0.0001	<0.0001	0.0007
	KLH	5.2	4.1	0.1531	<0.0001	0.0050
	NCD	6.9	6.6	0.4984	0.0482	0.2826
	IBD	3.1	2.4	0.1337	<0.0001	0.1975
Corticosterone (ng/ml)		2.9	3.0	0.8372	0.0517	0.8177

[1] Data on feed intake and hen-day egg production were divided into 4 periods; P1 is the adaptation period (d -11 to 0), P2 is the first week of the experimental period (d 1 to 7), P3 is the second and third wk of the experimental period (d 8 to 22), and P4 is the recovery period after the end of heat stress (d 23 to 29).

[2] The BW development in time was calculated compared to BW at d 5 prior to the start of heat stress.

EARLY-LIFE EXPERIENCE WITH COMBINED HEAT EXPOSURE AND LPS ADMINISTRATION

Levels of Ab binding to LPS were increased after combined exposure to heat and LPS. Hens with early-life heat and LPS experience had a different response level and response pattern compared to hens exposed to heat and LPS only at adult age (Treatment × Time interaction, $P < 0.01$; Table 7.4; Figure 7.1B). Levels of Ab binding to LPS were comparable between hens exposed to combined heat and LPS and hens only administered with LPS, indicating that heat stress had no additive effect on levels of Ab binding to LPS compared to LPS treatment only.

Levels of natural Ab binding to KLH were increased after combined exposure to heat and LPS. Hens with early-life heat and LPS experience had a different response pattern compared to hens exposed to heat and LPS only at adult age (Treatment × Time interaction, $P < 0.01$; Table 7.4; Figure 7.2B). Hens with early-life experience exposed to combined heat and LPS or only LPS had comparable levels of natural Ab binding to KLH. Hens without early-life experience and exposed to combined heat and LPS had lower levels of natural Ab binding to KLH at d 28 after the start of heat stress compared to hens without early-life experience and only administered with LPS (Treatment × Time interaction, $P < 0.05$).

During P3, feed intake was lower for hens with early-life experience to heat and LPS compared to hens that were exposed to heat and LPS only at adult age ($P < 0.05$, Table 7.4). Hens with early-life experience to heat and LPS, however, had already a lower feed intake during P1. Between P1 and P3, feed intake decreased by 26.4% in hens with early-life experience to heat and LPS, and by 24.8% in hens that were exposed to heat and LPS only at adult age.

No differences were found for BW development in time, hen-day egg production, egg weight, clutch length, level of Ab binding to NCD and IBD, complement activity of CPW, APW, and MBL, and corticosterone level between hens with early-life experience to heat and LPS and hens that were exposed to heat and LPS only at adult age (Table 7.4). Seven laying hens died within 1 d after LPS administration combined with heat exposure at 24 wk of age; 2 laying hens with early-life experience to heat and LPS died, and so did 5 laying hens that were exposed to heat and LPS for the first time.

DISCUSSION

In the present study, effects of early-life experience with single or combined climatic (heat) and hygienic (LPS) stressors on performance, humoral immune competence, and endocrine

responsiveness after re-exposure to a similar stressor in later-life were investigated in laying hens. The purebred layer line used to test the hypothesis has been used in previous studies (Star et al., 2007b,2008a,b), where adult hens were exposed to single or combined climatic stress (heat) and hygienic stress (LPS) during early lay. Hens of this line were not able to maintain a high hen-day egg production during heat stress and showed more fluctuations in humoral immune responsiveness compared to 3 other purebred layer lines, what might indicate that the adaptability of this line to the stressors was suboptimal and therefore a good candidate to study the possibility to improve the adaptability of laying hens.

All performance, immune, and endocrine parameters were only measured in adult laying hens at similar time points as in previous studies (Star et al., 2007b,2008a,b). Responses to heat stress within the present and previous studies were comparable for performance parameters; feed intake, BW, hen-day egg production, and egg weight decreased. The aim of the present study was, however, to improve adaptability to heat stress by early-life exposure, where adaptability can be defined as an individuals' ability to change feed intake, production, behaviour, and other features of live in response to environmental changes. In broilers, several studies have been performed (Arjona et al., 1990; De Basilio et al., 2001; Yahav and Hurwitz, 1996; Yahav and Plavnik, 1999; Yahav and McMurtry, 2001) showing improved adaptability to heat stress in later-life, e.g., improved thermo tolerance and reduced mortality when exposed to heat stress for 24 h at d 5 of hatch. In most of these studies, it was concluded that thermal conditioning resulted in improved performance at marketing age, based on growth, feed intake, and feed efficiency between the period of early-life heat exposure and later-life heat exposure. In the present study, however, early-life exposure to heat stress did not improve the adaptability of laying hens during re-exposure to heat stress in later-life, based on these performance parameters. There are several possible explanations why early-life heat exposure in layers did not result in the expected improvement of adaptability. First, laying hens may have a sufficient thermoregulatory system irrespective of early-life heat exposure. Second, there is a difference between broilers and layers in growth rate and, related to this, in heat production. Broilers have a higher heat production and are therefore more susceptible to heat stress. Third, time between first and second heat exposure was about 23 wk, which is a long period compared to the 4 to 5 wk between the first and second heat exposure in broilers. This might suggest a flow of memory underlying adaptability to stress in time. Fourth, during re-exposure to heat stress in the present study, temperature was lower (32ºC) and time of exposure was longer (22 d) compared to studies done in broilers where temperature was

(above) 35ºC for only 6 to 8 h. Fifth, the period of early-life heat exposure was based on a physiological 'sensitive' period for imprinting, however, this might not be the right period to manipulate the thermoregulatory system of layers. Even early thermal manipulation during incubation, may alter thermo tolerance at later age as can be hypothesized from studies of Moraes et al. (2003,2004) and Yahav et al. (2004a,b) in broilers and of Janke et al. (2002) in laying hens. However, it is still unclear if we deal here with similar effects of thermal conditioning.

Responses to LPS administration within the present and previous studies were comparable for performance parameters; feed intake, BW, hen-day egg production, and egg weight were decreased in the first wk after LPS administration. Although early-life LPS exposure did not improve the performance of laying hens re-exposed to LPS in later-life, early-life LPS exposure significantly influenced the kinetics and magnitude of the response of Ab binding to LPS in later-life. Hens administered with LPS only at adult age showed an immune response with primary characteristics with a peak in Ab level at d 7 after LPS administration, whereas hens with early-life LPS experience had an accelerated and higher peak in Ab level at d 4 after LPS administration and showed a secondary immune response. The observed change in the kinetics and magnitude by early-life LPS exposure indicated a classic T-cell dependent Ab response (memory) to LPS, as also observed after intra-tracheally administered LPS in laying hens (Ploegaert et al., 2007). This is, however, in contrast with the established concept that Ab responses to LPS are T-cell independent [as mentioned for instance by Gehad et al. (2002)] and, therefore, no memory to LPS should be developed.

In the previous studies, it has been shown already that levels of natural Ab binding to KLH increased after LPS administration, and the present study showed that early-life LPS exposure influenced the kinetics of the response of natural Ab binding to KLH. Hens with early-life LPS experience had a peak in natural Ab level to KLH at d 4 after LPS administration and hens administered with LPS only at adult age had a peak in natural Ab level to KLH at d 7 after LPS administration. It is not clear yet, why natural Ab binding to KLH are enhanced after LPS challenge. It seems, however, that the response is B-cell related and that the change in kinetics is based on B-cell memory.

In the present study, a 2.5–fold increase in activity of MBL was induced 1 d after LPS administration (data not shown). Other studies (Juul-Madsen et al., 2003; Laursen and Nielsen, 2000) also found an up-regulation of MBL activity in chickens during the acute stages of infections. These studies confirm that MBL can bind to LPS and initiates a pro-inflammatory response (Kang et al., 2007). In the present study, however, there was no

difference in the up-regulation of MBL activity between hens with early-life LPS experience and hens only administered with LPS as adults. This indeed suggests that binding of LPS by MBL initiates a pro-inflammatory response irrespective of any experiences with LPS, indicating the short-term severity of this endotoxin. In addition, the short-term severity of LPS might be the reason why no effect was observed on performance parameters and hens did not become refractory to repeated LPS administrations, which is in contrast to the findings by Korver et al. (1998) and Parmentier et al. (2006).

Effects of combined exposure to heat and LPS in hens with early-life experience and hens only exposed at adult age were comparable with effects observed after single LPS administration in hens with early-life LPS experience and hens only administered at adult age, respectively, indicating that heat stress had no additive effect.

The present data revealed no positive effect of early-life heat stress exposure on the adaptability of laying hens in later-life. However, further research, focussed on pre- versus postnatal heat exposure is recommended. Furthermore, early-life LPS exposure and combined early-life exposure to heat and LPS did not affect adaptability in terms of performance and endocrine responsiveness. Early-life LPS exposure and combined early-life exposure to heat and LPS, however, had comparable effects on the kinetics and magnitude of (natural) Ab levels binding to LPS and to KLH, indicating that early-life LPS exposure can enhance the status of immune reactivity or induce a higher sensitivity to LPS.

ACKNOWLEDGMENTS

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CHAPTER 8

General discussion

INTRODUCTION

There is a limited number of internationally operating poultry breeding companies that provide laying hens worldwide. For these companies, it is favorable to have laying hens that can function under a variety of environmental conditions. To ensure an appropriate level of performance, health, and welfare of their laying hens, breeding companies need 'robust' laying hens. Robustness is a term which is rapidly becoming a main interest in animal production (Knap, 2005), and can be implemented on different levels in the animal production chain, e.g., chain level, system level, farm level, and animal level.

In association with breeding company Hendrix Genetics, the project 'The genetics of robustness in laying hens' was started. The aim of the project was to investigate nature and regulation of robustness in laying hens under sub-optimal conditions and the possibility to increase robustness by using animal breeding without loss of production. The experiments described in this thesis investigated parameters that could predict the robustness of purebred layer lines, where the influence of genetic background, environmental conditions, and early-life experiences was used as framework. The major findings of the experiments will be briefly discussed in this Chapter. The focus of this Chapter, however, will be on robustness.

DEFINITION OF ROBUSTNESS

In general, robustness is a property that allows a system to maintain its functions despite external and internal perturbations. Robustness is often misunderstood to mean staying unchanged regardless of stimuli or mutations, so that the structure and components of the system, and therefore the mode of operation, is unaffected. In fact, robustness is the maintenance of specific functionalities of the system against perturbations, and it often requires the system to change its mode of operation in a flexible way. In other words, robustness allows changes in the structure and components of the system owing to perturbations, but specific functions are maintained (Kitano, 2004).

Also for organisms, robustness is a property that allows the organism to maintain its functions despite external and internal influences with the ultimate goal to survive and reproduce. Every organism alive today is the product of many generations in which its progenitors managed to produce progeny that survived to reproduce. To achieve this consistency, organisms must have a balance between robustness and evolvability (De Visser et al., 2003; Earl and Deem, 2004). Evolution often selects traits that might enhance

robustness of the organism. Robustness is, therefore, ubiquitous in living organisms that have evolved (Kitano, 2004).

Biological robustness, as described above, seems to cover a broad scale of biological systems, including robustness on the level of the organism. Robustness on the level of the organism is of interest in animal production, and therefore robustness has to be defined in this context. Different animal scientists will define robustness in comparable terms that are common grounded, but not exactly the same (Box 8.1).

Box 8.1. Definitions of robustness by animal scientists.

- An animal under a normal physical condition that has the potential to keep functioning and take short periods to recover under varying environmental conditions (this thesis).
- The coping potential of an animal to survive in a multi stressor environment.
- Undisturbed functioning of an animal in a suboptimal environment.
- To maintain a high level of production and high level of health under various environmental conditions.
- The plasticity or flexibility of an animal to cope with a broad set of environmental factors with the aim to survive.
- Undisturbed functioning, also under suboptimal circumstances as climate, feeding, or disease pressure.
- The possibility of an animal to react adequately to multiple stressors, i.e., with highest priority for 'life traits' and survival.

Although these definitions are not the same, the main concept of robustness in animal production seems to be more clear: to keep functioning under various (suboptimal) environmental conditions with the aim to survive and to maintain a high production level. This is in accordance with the more general interpretation of Kitano (2004) who stated that robustness allows changes in the structure and components of the system owing to perturbations, i.e., a robust animal has to be flexible, but specific functions are maintained, e.g., survival and reproduction.

From an evolutionary point-of-view, natural selection favors switch mechanisms that trade-off resources among the competing demands of survival and reproduction (Figueredo et al., 2006). The Life History Theory is an evolutionary model that describes a continuum ranging from species that follow a 'producer' strategy to species that follow a 'survivor' strategy (Ellis et al., 2006).

In the next paragraph, the Life History Theory will be explained in more detail. Based on this theory, a basic model for robustness in animal production, hereafter mentioned as the

Robustness model, will be proposed. The Robustness model will focus on the basic fitness elements survival and reproduction, with the idea that robustness is not a continuum with the extremes in survival and reproduction as stated by the Life History Theory, but that interactions between survival and reproduction occur. To my opinion, because of these interactions, the model has to be extended.

ROBUSTNESS: SURVIVAL, REPRODUCTION, AND FLEXIBILITY

Life History Theory and the Robustness model

Life History Theory (reviewed by Figueredo et al., 2006) is the basis of a number of studies describing relationships among pace of maturation, length of lifespan, reproductive effort, and degree of social cohesion. The r/K continuum proposed by the Life History Theory (Box 8.2) represents a covarying range of reproductive behavior patterns inversely relating life history traits such as fertility and parental investment. The endpoints of this continuum range from extreme r (e.g., maximum egg output and no parental care) to extreme K (e.g., minimal birth rate and elaborate parental care).

Box 8.2. The r/K selection theory.

In ecology, the r/K selection theory relates to the selection of traits which promotes success in particular environments. The theory originates from work on island biogeography by the ecologists MacArthur and Wilson (1967). In the r/K selection theory, selective pressures are hypothesized to drive evolution in one of two generalized directions: r- or K-selection (Pianka, 1970). These terms, r and K, are derived from standard ecological algebra, where r is the growth rate of the population, and K is the carrying capacity of its local environmental setting.

To illustrate the conceptual differences in life history strategies, it is instructive to compare r-selected species, which preferentially allocate metabolic resources to reproduction, with K-selected species, which preferentially allocate metabolic resources toward growth, maintenance, and survival (Ellis et al., 2006).

- r-selected species evolved under unstable and unpredictable conditions, leading to a strategy focusing on the *production* of individuals (i.e., offspring quantity). Rabbits, for example, exhibit rapid sexual development, high fertility, low parental investment, high infant mortality, low interbirth interval, short lives, generally small size, less

group cohesion, and less competition for resources because, historically, they evolved under unstable conditions where short-term strategies paid off.

- K-selected species evolved under stable and predictable conditions, leading to a strategy focusing on the *survival* of individuals (i.e., offspring quality). Elephants, for example, exhibit slow delayed sexual development, low fertility, high parental investment, low infant mortality, high interbirth interval, greater longevity, generally large size, high group cohesion, and intense competition for resources because, historically, they evolved in stable environments where long-term strategies paid off.

Generally, chickens appear to be r-selected. Sexual development of chickens is rapid, they are highly fertile, they produce a number of offspring at a time, they suffer high infant mortality, and given multiple predation risks they may have little ability to protect their offspring and thus there is little opportunity for high levels of parental care to evolve. Poultry production has taken advantage of these characteristics; laying hens start lay at an early age and they produce on average 300 eggs per hen per year (Preisinger and Flock, 2000; Chapter 2).

The Life History Theory as described above, is based on variation between species. However, within species there is a substantial degree of individual variation in the life history strategy, which can be contributed to individual difference regarding genetic and environmental conditions (reviewed by Figueredo et al., 2006). An assumption of the Life History Theory is, therefore, that the same divergent environmental conditions that favor the evolution of r-selected versus K-selected reproductive strategies between species also favor the development of alternative reproductive strategies within species. The symbols r and K will be used than as heuristics to refer to alternative reproductive strategies within populations that roughly approximates variation on the r/K continuum (Ellis et al., 2006).

The purebred layer lines used in our studies are mainly selected for high reproduction and survival. Still, the lines differ in reproduction level and survival rate. Based on the Life History Theory, we might be able to distinguish two patterns of phenotypic variation that are rough approximations of the r and K life history strategies in these layer lines (Figure 8.1A). From an evolutionary point-of-view, it is obvious that survival and production are the basic fitness elements, and these specific functions have to be maintained. This implies that it is biologically not possible to transform a ‘survivor’ into a ‘producer’ or vice versa. It is possible, however, to take advantage of the dynamics in these strategies, as already done by breeding for reproduction. Robustness of an animal can be linked to the biological robustness as proposed by the Life History Theory. Assuming that interactions between

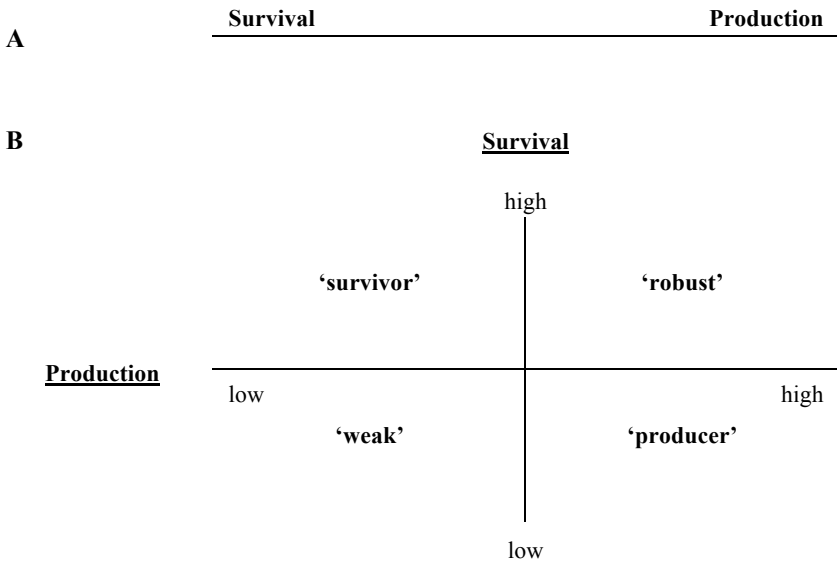

Figure 8.1. [A] Survival and reproduction strategies based on the Life History Theory, [B] four strategies in the Robustness model that can be distinguished by assuming interactions between survival and reproduction and by integrating this interaction in the Life History Theory.

survival and reproduction occur, the Life History Theory can be extended and robustness can be included in the model, where the most important characters of a robust animal are a high reproduction and high survival rate. By including the interaction between survival and reproduction, e.g., robustness, into the Life History Theory, 4 different strategies are possible: 'producers' (r-selected), 'survivors' (K-selected), 'robust' animals, and opposite of robust animals are 'weak' animals, i.e., animals with a low reproduction and low survival rate. These 4 strategies form the basis of the Robustness model and are illustrated in Figure 8.1B.

By ranking the lines for survival rate and reproduction level, a first impression of different strategies within species can be obtained (Figure 8.2). Layer lines were classified as 'survivor' or 'producer' as strategies of the Life History Theory (Figure 8.2A). Ten layer lines could be classified as 'survivor' or 'producer'. Line BE and WA had the same rank number and were in-between the 'survivors' and 'producers'. However, line BE has a low production and low survival rate, and line WA has a high production and high survival rate, which makes it hard to classify them as 'survivor' or 'producer'.

Second, the lines were classified as 'survivor', 'producer', 'robust', or 'weak' as strategies of the Robustness model (Figure 8.2B). Based on this ranking, 8 of the 12 lines could be classified as 'survivor' or 'producer'. Line BE and WA, that could not be classified by the Life History Theory, were classified by the Robustness model as 'weak' and 'robust', respectively. Furthermore, line BB was classified as 'weak' and line WF was classified as 'robust'. Noteworthy, most of the Rhode Island Red layers have a lower reproduction, so within species there was an obvious difference in reproduction between Rhode Island Red and White Leghorn layers.

A

Survival		**Production**
B2, B3, BA, BB, WD	BE, WA	B1, W1, WB, WC, WF

B

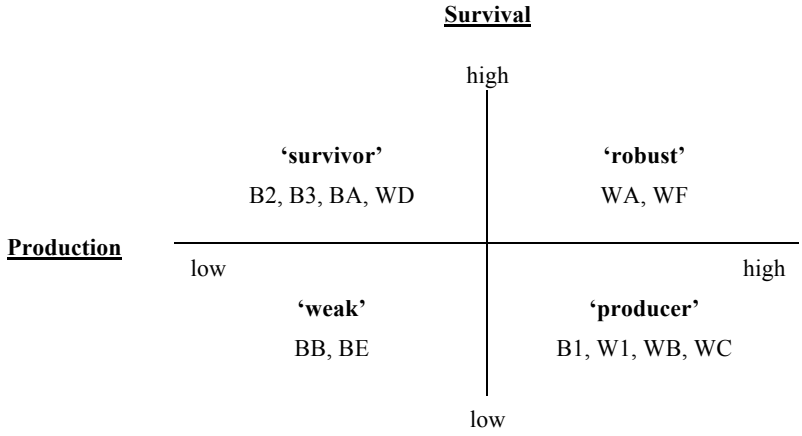

FIGURE 8.2. [A] Classification of twelve purebred layer lines into two strategies of the Life History Theory, [B] classification of twelve purebred layer lines into four strategies of the Robustness model, based on interaction between survival (from 20 to 72 weeks of age) and reproduction (from 25 to 69 weeks of age). Lines were ranked from 1 to 12 for reproduction and survival, where the highest production was ranked as '12' and the highest survival was ranked as '1'. In this way, 'survivors' that have a high survival rate and low production will have a low total rank number, and 'producers' that have a low survival rate and high production will have a high rank number.

Immune defense as potential key factor for survival and reproduction strategies

Immune defenses can affect host resources influencing growth, reproduction (Bonneaud et al., 2003; Klasing and Korver, 1997), and survival (Armitage et al., 2003). Immune responses can differ between species, but also between breeds of domesticated species. Vertebrate species have a complex immune system, and predicting which immune defense strategy favors survival or reproduction is difficult (Lee and Klasing, 2004). With respect to evolutionary development of the immune system, however, the innate immune system is of interest. Innate (natural) immunity is the most universal, rapid acting and, probably, most important part of immunity; most organisms survive through innate immune systems alone (Beutler, 2004). But also the innate immune system, as a topic for investigation, is enormously broad. In part, this is because innate immune mechanisms are dynamic on an evolutionary time scale. The host population is shaped by the selective pressures that pathogens impose, and survives as best as it can (Beutler, 2004).

As mentioned in Chapter 1, the innate immune system can be divided in several components, like natural antibodies and complement as studied in this thesis (Chapter 3 to 7). In this paragraph, the focus will only be on natural antibodies (NAb).

Natural antibodies are defined as antigen-binding antibodies present in non-immunized individuals, which have a broad specificity repertoire and usually a low binding affinity, and which can be directed to exogenous as well endogenous antigens (Boes, 2000; Ochsenbein et al., 1999). Furthermore, Lee and Klasing (2004) proposed that increased antibody-mediated defense, including constitutive levels of circulating antibodies (NAb) and induced (specific) antibody responses, are likely to be the most successful strategy of an organism for dealing with novel pathogenic challenges. Thus, NAb are potentially important biological agents and prevalent in the healthy immune repertoire (Bayry et al., 2005). An ineffective level and low diversity of NAb is a serious risk factor for health and survival, because an ineffective level or low diversity of NAb causes a delayed specific immune response resulting in increased mortality (Baumgarth et al., 1999; Harada et al., 2003; Jayasekera et al., 2007).

We have established a predictive value of the level of NAb binding to keyhole limpet haemocyanin (KLH) in relation to survival (Star et al., 2007a; Chapter 3). Low levels of NAb binding to KLH were detected in chickens that not survived the laying period. Because KLH is a ‘classical’ antigen for NAb, it is supposed that NAb binding to KLH reflects the capacity to mount an appropriate level of natural immune defense. Although, the established relation between low levels of NAb binding KLH and the probability to

survive was regardless of layer line, a distinction could be made between lines showing high or low immune competence (with respect to NAb, complement activity, and specific antibodies), and also between lines showing high or low survival rates. In the context of Life History Theory, r-selected 'producers' are more vulnerable to, at least, stress-induced illness, where K-selected 'survivors' are more resistant to stress-induced illness (reviewed by Ellis et al., 2006). Therefore, we expect that purebred layer lines that are defined as 'producer' have lower levels of NAb binding KLH besides higher production levels and lower survival rates, whereas lines that are defined as 'survivor' have higher levels of NAb binding KLH besides lower production levels and higher survival rates. From 'robust' animals we expect them to have higher levels of NAb binding KLH, and higher production levels and survival rates.

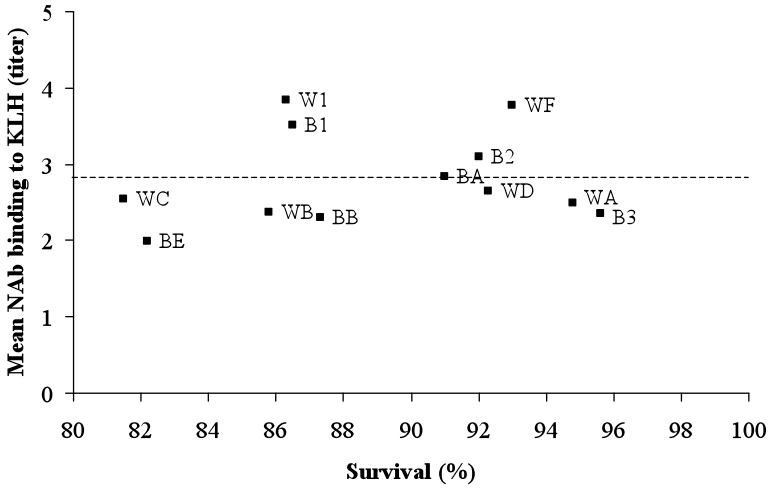

FIGURE 8.3. Survival (%, from 20 to 72 weeks of age) and level of natural antibodies (NAb) binding to keyhole limpet haemocyanin (KLH; mean of levels measured at 20, 40, and 65 weeks of age) in twelve purebred layer lines. [---] shows the overall mean level of NAb binding to KLH (titer is 2.8).

First, the level of NAb binding to KLH and survival rate or egg production of each of the twelve layer lines is shown in Figure 8.3 and 8.4, respectively (data from Chapter 3). Figure 8.3 shows a clear distinction between 'survivor' and 'robust' lines on the one hand, and 'producer' and 'weak' lines on the other hand, whereas Figure 8.4 shows a clear distinction between 'producer' and 'robust' lines on the one hand, and 'survivor' and 'weak' lines on the other hand. However, concerning the level of NAb binding to KLH, no clear distinction

can be made between lines that were classified as 'survivor', 'producer', or 'robust'. According to the expectations, based on classification by the Robustness model, the two lines that were classified as 'weak', BB and BE, have a low level of NAb binding to KLH.

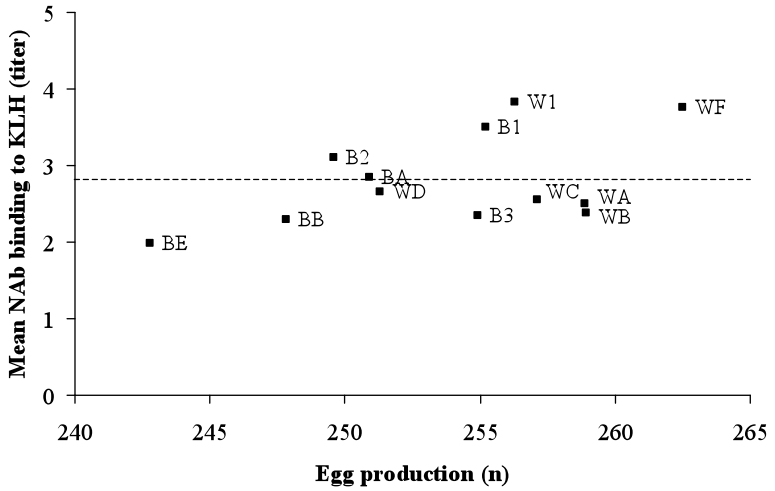

Figure 8.4. Egg production (n, from 25 to 69 weeks of age) and level of natural antibodies (NAb) binding to keyhole limpet haemocyanin (KLH; mean of levels measured at 20, 40, and 65 weeks of age) in twelve purebred layer lines. [---] shows the overall mean level of NAb binding to KLH (titer is 2.8).

Second, lines were classified based on ranking for survival rate, production level, and level of NAb binding to KLH (Table 8.1). Based on this ranking, 8 of the 12 lines can be classified as 'survivor', 'producer', 'robust', or 'weak'. Lines B2, BA, and WD can be classified as 'survivor', i.e., lines with low production, high survival rate, and high level of NAb. Lines WB and WC can be classified as 'producer', i.e., lines with a high, production, low survival rate, and low level of NAb. Line WF is the only line with a high production level, high survival rate, and high level of NAb binding to KLH, and therefore this line can be classified as 'robust'. The two lines that can be classified as 'weak' are lines BB and BE, which is comparable with the classification based on survival and production only (Figure 8.2B). Interestingly, a line with a low production, low survival rate, and high NAb level does not exist in this model. Evolutionarily, this would be a line with low fitness, because it has a low production level, a low survival rate, but it makes an effort to its immune system. For the same reasons, such a line with low fitness would not be of interest for poultry production.

TABLE 8.1. Ranking of the twelve purebred layer lines for high or low survival rate, production level[1], and innate immune competence[2]

Egg production	Innate immune competence	Survival rate	
		High	Low
High	High	WF	B1, W1
	Low	WA	WB, WC
Low	High	B2, BA, WD	-
	Low	B3	BB, BE

[1] Based on egg production from 25 to 69 weeks of age.

[2] Based on natural antibody levels binding to keyhole limpet haemocyanin at 20, 40, and 65 weeks of age.

Summarizing, classification of the Robustness model is based on the interaction between the basic fitness elements survival and reproduction. Based on the Robustness model layer lines can be classified as 'survivor', 'producer', 'robust', or 'weak'. There is strong evidence that survival is related to the level of natural antibodies. Based on the combination of reproduction, survival, and level of natural antibodies, one layer line can be classified as 'robust', namely line WF.

FLEXIBILITY

As already mentioned, robustness of an animal depends on the one hand on maintaining survival and production, and on the other hand on being sensitive to unpredictable fluctuations in the environment, i.e., a robust animal has to be flexible.

First evidence that lines differ in flexibility comes from the variability in production as measured in the experiment described in Chapter 3. Data on production was not described in that Chapter, but production per cage (4 hens per cage) was registered during the laying period for each of the 12 layer lines. In Figure 8.5, the variability (SD) in production is given for 4 lines. These lines were also described in Chapter 4 to 6.

Variability in production in this Figure is based on one laying period, where the layer lines were housed in the same housing facility under commercial circumstances. The laying hens were therefore exposed to similar unpredictable environmental influences during the laying period. Differences in variability in production can be attributed to sensitivity towards environmental influences or to genetic differences. It appears, however, that lines WF and WA, classified as 'robust', showed less variability in production, than lines B1 and WB that were classified as 'producer'. This is contradictorily with the expectation that layer

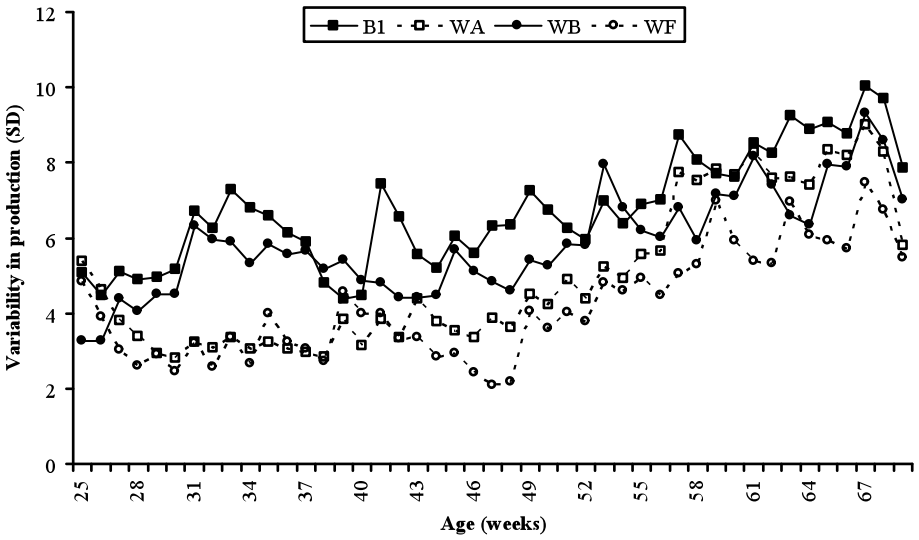

FIGURE 8.5. Variability in production (SD per week) registered from 25 to 69 weeks of age for 4 genetically different purebred layer lines.

lines classified as 'robust' will react in a more flexible way, i.e., will have a higher variability in production, than layer lines classified as 'producer'.

Second evidence that these lines differ in flexibility comes from the experiments described in Chapter 4 to 6. To evaluate flexibility of layer lines under challenging environmental conditions, 4 of the 12 layer lines were selected. The lines were characterized by low or high survival rate and low or high innate immune competence (Figure 8.3). Each of these 4 lines was also classified as a high producing line, i.e., as 'producer' or 'robust' in the Robustness model. The 4 selected layer lines were exposed to environmental stressors: heat stress (32ºC), challenge with lipopolysaccharide [LPS (1 mg/kg of body weight of *Escherichia coli* LPS)], or a combination of both stressors.

The results of these experiments are described in Chapter 4 to 6. In these Chapters, however, the focus was on the effects of line, temperature challenge, and LPS challenge, rather than flexibility of the lines towards these stressors, although the first step towards flexibility was taken (Chapter 6). Here, flexibility will be evaluated and discussed in more detail.

To give a first impression of flexibility, NAb binding to KLH for each of the 4 layer lines in a control situation, in a heat stress situation, after LPS challenge, or by combined exposure to heat and LPS, is given in Figure 8.6. The control situation (Figure 8.6A) clearly

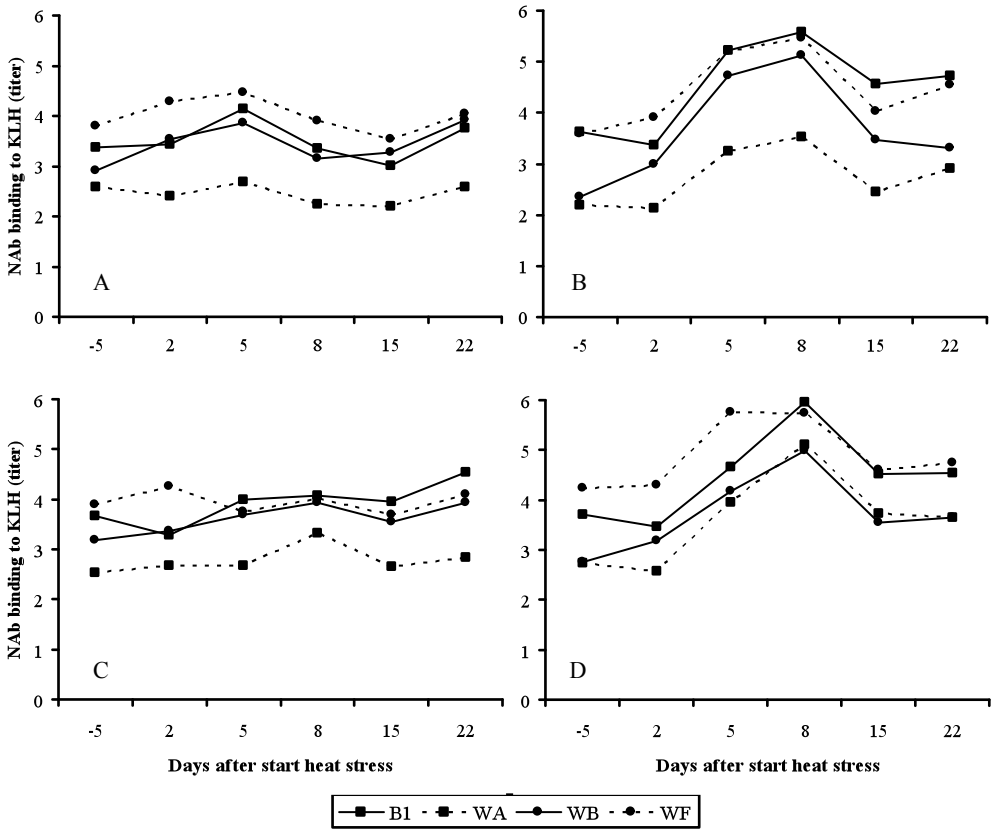

FIGURE 8.6. Recovery of natural antibodies (NAb) binding to keyhole limpet haemocyanin (KLH) after exposure to heat stress, LPS challenge, or combined exposure in 4 purebred layer lines. [A] control treatment, [B] LPS challenge (1 mg/kg of BW of *Escherichia coli* LPS) at d 1 after the start of heat stress, [C] continuous heat exposure (32ºC), start at d 0, [D] combined exposure to heat and LPS.

shows a line difference in level of NAb binding to KLH, but also a 'natural' fluctuation in NAb level over time for each line. After LPS challenge (Figure 8.6B), each line showed an increase in NAb binding to KLH. These figures give an impression of line and treatment effects, and of responsiveness towards the environmental stressors. However, responsiveness probably does not depend on one parameter, and we like to give an overview of the responsiveness towards environmental stressors of each of the fourteen parameters as described in Chapter 4 to 6. Therefore, the responsiveness of each line, for each parameter, per treatment, is expressed as [↕] for high responsiveness and as [↔] for

Table 8.2. Responsiveness of 4 purebred layer lines for different parameters per treatment (LPS challenge, exposure to heat stress, or combined exposure to LPS and heat stress). Responsiveness was determined by taking the mean of the measurements after the start of heat stress and this was compared with the mean of the measurements before start of the heat stress. The difference between mean measurement before and after the start of heat stress represents the responsiveness. Responsiveness is expressed as [↕] for high responsiveness and [↔] for low responsiveness. Overall responsiveness for each line per treatment is expressed as the number of high responsive arrows ([↕]) compared to the number of parameters (fourteen in total).

	LPS				Heat stress				LPS and heat stress			
	B1	WA	WB	WF	B1	WA	WB	WF	B1	WA	WB	WF
Performance												
Feed intake	↔	↕	↕	↔	↕	↕	↔	↔	↕	↕	↔	↔
Body weight	↔	↔	↕	↕	↕	↕	↔	↔	↕	↕	↔	↔
Egg production	↕	↔	↕	↔	↕	↕	↔	↔	↕	↕	↔	↔
Egg weight	↔	↕	↕	↔	↕	↕	↔	↔	↕	↔	↕	↔
Immunity												
NAb KLH	↕	↔	↕	↔	↔	↕	↕	↔	↔	↕	↕	↔
Ab LPS	↔	↔	↕	↕	↔	↕	↕	↔	↔	↕	↔	↕
Ab HuSA	↔	↕	↕	↔	↕	↕	↔	↔	↕	↕	↔	↔
CPW	↕	↔	↕	↔	↕	↔	↕	↔	↕	↔	↕	↔
APW	↔	↕	↕	↔	↕	↕	↔	↔	↕	↔	↔	↕

Table 8.2, continued. Responsiveness of 4 purebred layer lines for different parameters per treatment [lipopolysaccharide (LPS) challenge, exposure to heat stress, or combined exposure to LPS and heat stress]. Responsiveness was determined by taking the mean of the measurements after the start of heat stress and this was compared with the mean of the measurements before start of the heat stress. The difference between mean measurement before and after the start of heat stress represents the responsiveness. Responsiveness is expressed as [↕] for high responsiveness and [↔] for low responsiveness. Overall responsiveness for each line per treatment is expressed as the number of high responsive arrows ([↕]) compared to the number of parameters (fourteen in total).

	LPS				Heat stress				LPS and heat stress			
	B1	WA	WB	WF	B1	WA	WB	WF	B1	WA	WB	WF
Endocrine and oxidative stress												
Corticosterone	↔	↔	↕	↕	↔	↕	↕	↔	↔	↕	↕	↔
T3	↕	↕	↔	↔	↔	↕	↕	↔	↕	↕	↔	↔
Glucose	↕	↕	↔	↔	↔	↕	↔	↕	↕	↕	↔	↔
Uric acid	↕	↕	↔	↔	↕	↔	↕	↔	↕	↕	↔	↔
TBARS	↕	↔	↕	↔	↕	↔	↕	↔	↔	↕	↕	↔
Overall responsiveness	7/14	7/14	11/14	3/14	9/14	11/14	7/14	1/14	10/14	11/14	5/14	2/14

Abbreviation key: NAb = natural antibody; Ab = antibody; SpAb = specific antibody; KLH = keyhole limpet haemocyanin; LPS = lipopolysaccharide; HuSA = human serum albumin; CPW = classical complement pathway; APW = alternative complement pathway; T3 = 3,5,3'-triiodothyronine; TBARS = thiobarbituric acid reacting substances.

low responsiveness (Table 8.2). The overall responsiveness for each line per treatment is expressed as the number of high responsive arrows ([↕]) compared to the number of parameters (fourteen in total).

Lines differ in responsiveness, and this partly depends on the type of environmental stressor (Table 8.2). Line WB is high responsive to challenge with LPS, whereas lines B1 and WA are high responsive to heat stress. When exposed to combined LPS and heat, the patterns in responsiveness slightly change. The effect of heat stress on responsiveness is, however, more explicitly presented than the effect of LPS challenge, resulting in an overall responsiveness that is comparable with exposure to single heat stress. Most intriguing is, however, that line WF expressed a low responsiveness to each environmental stressor, and to almost each of the parameters. As for the variability in reproduction, this again, is in contradiction with the expectation that a layer line classified as 'robust' will react in a more flexible way, i.e., will have a higher responsiveness to environmental stressors, than a layer line classified as 'producer'. Although the strategies, based on survival, reproduction, and flexibility, are not always consistent, it is clear that line WF is able to maintain a very stable strategy in performance, immunity, and endocrinology, contributing to a high survival rate and high production.

In Chapter 6, it was suggested that stability in performance and immunity was regulated by sensitivity in the glucocorticoid hormone corticosterone. This suggestion was based on the sensitivity in corticosterone of line WB in response to the stressors resulting in a more stable production and less sensitive specific immune response to human serum albumin (HuSA), and less sensitivity in corticosterone of line B1 in response to the stressors resulting in a less stable production and more sensitive specific immune response to HuSA. This suggestion still stands under heat stress conditions for the difference in response levels between these two lines. However, it does not explain the stable performance and specific immunity of line WF in combination with less sensitivity in corticosterone under heat stress condition. Furthermore, it does not explain the high overall responsiveness of line WB and the low overall responsiveness of line WF after LPS challenge, while both lines had a high responsiveness in corticosterone after LPS challenge.

To start with the second remark, that both line WB and WF had a high responsiveness in corticosterone after LPS challenge, while line WB had a high overall responsiveness and line WF a low overall responsiveness. Responsiveness of the 4 purebred layer lines is posed as high versus low, where 2 lines are characterized as high responsive and 2 lines as low responsive. For corticosterone, however, the difference in responsiveness between the 4 lines is very small. This, together with the non-significant effect of LPS on corticosterone

level (Chapter 6), indicates that responsiveness in corticosterone level is not informative for the flexibility towards LPS challenge between the 4 purebred layer lines.

Concerning the first remark, that both line WB and WF had a low responsiveness in performance and specific immunity, while line WB had a high responsiveness in corticosterone and line WF a low responsiveness. Responsiveness in level of corticosterone is affected by heat stress (Chapter 6), but seems not indicative for performance and (specific) immunity under heat stress conditions. Related to this, is that the heat stress period lasted for three weeks, while the main effects of corticosterone can be expected shortly after start of the heat stress.

We can speculate which parameter is a better indicator for flexibility, but a complex phenomenon as flexibility is probably hard to determine by only one parameter. Therefore, an overall responsiveness based on a number of parameters (as shown in Table 8.2) is a better indicator for flexibility. Based on this overall responsiveness, it can be concluded that flexibility depends on genotype and the type of environmental stressor, where line WF is low responsive to both heat stress and LPS challenge, indicating that robustness can be associated with low responsiveness.

EFFECT OF EARLY-LIFE EXPERIENCE ON FLEXIBILITY

Robustness of an animal depends on genetic potential and environmental influences that hamper or facilitate adaptive responses. The responsiveness of animals to environmental influences, however, can be acquired and improved, i.e., animals can be conditioned to an unexpected change in the environment. Such a conditioning process, preferably during early-life, may be used to prepare animals to unexpected heat spells or changes in hygienic circumstances in later-life. The aim of the experiment described in Chapter 7 was, therefore, to improve adaptability to heat stress or LPS challenge by early-life exposure, where adaptability can be defined as the ability of an individual to change feed intake, production, behaviour, and other features of live in response to environmental changes.

Line B1 was chosen for this experiment, mainly because this line was not able to maintain a high hen-day egg production during heat stress. However, early-life heat stress experience and early-life LPS experience did not affect adaptability of these laying hens towards heat stress or LPS challenge in later-life, e.g., no difference in feed intake, body weight, hen-day egg production, and egg weight. Early-life LPS experience did affect, however, kinetics and magnitude of Ab levels binding to LPS and KLH. It was suggested that the observed change in kinetics and magnitude in Ab levels binding to LPS is T-cell dependent and based on T-cell memory, and that the observed change in kinetics in NAb

levels binding to KLH is B-cell related and is based on innate receptors on B-cells maintaining a form of non-specific memory. This suggestion was further investigated by measuring isotype responses (IgM and IgG). In general, IgM is the major immunoglobulin produced during a primary response (induced by B cells) and binds antigens relatively less specific, whereas IgG is the major immunoglobulin produced during a secondary response (induced by T cells) and binds antigens more specific. The results are shown in Table 8.3 and Figure 8.7.

Table 8.3. Effect of single (old) or repeated (young + old) challenge with *Escherichia coli* lipopolysaccharide (LPS) on antibody level binding to LPS and keyhole limpet haemocyanin (KLH) specified by isotype IgM and IgG

Isotype	Young + old	Old	ANOVA		
			Treatment	Time	Treatment × Time
LPS					
IgM	3.8	4.3	0.0043	<0.0001	<0.0001
IgG	10.6	8.5	<0.0001	<0.0001	<0.0001
KLH					
IgM	7.9	7.9	0.9967	<0.0001	0.0154
IgG	4.7	4.6	0.6442	<0.0001	0.0007

These results show a T-cell dependent LPS response, indicated by a significant higher IgG and lower IgM level in laying hens with early-life LPS experience compared with hens administered with LPS only at adult age (Figure 8.7A), and reveal a B-cell related KLH response after LPS challenge, indicated by a change in the kinetics of IgM and IgG (Treatment × Time interactions; Figure 8.7B). The observed change in kinetics and magnitude of LPS-IgM and LPS-IgG by early-life LPS exposure indicated a classic T cell-dependent Ab response (memory) to LPS, which is in contrast with the established concept that Ab responses to LPS are T cell-independent and that no memory to LPS should be developed. Together, these results on antibody level, indicate that early-life LPS exposure can enhance the status of immune reactivity or induce a higher sensitivity to LPS in later-life. The influence of an enhanced status of immune reactivity or higher sensitivity to LPS on disease resistance has to be investigated, but might be of interest for lines with a genetically low level of innate immune competence, like line WB.

Summarizing, the basic elements for robustness are survival and reproduction. Furthermore, robustness can be indicated by sensitivity to unpredictable fluctuations in the

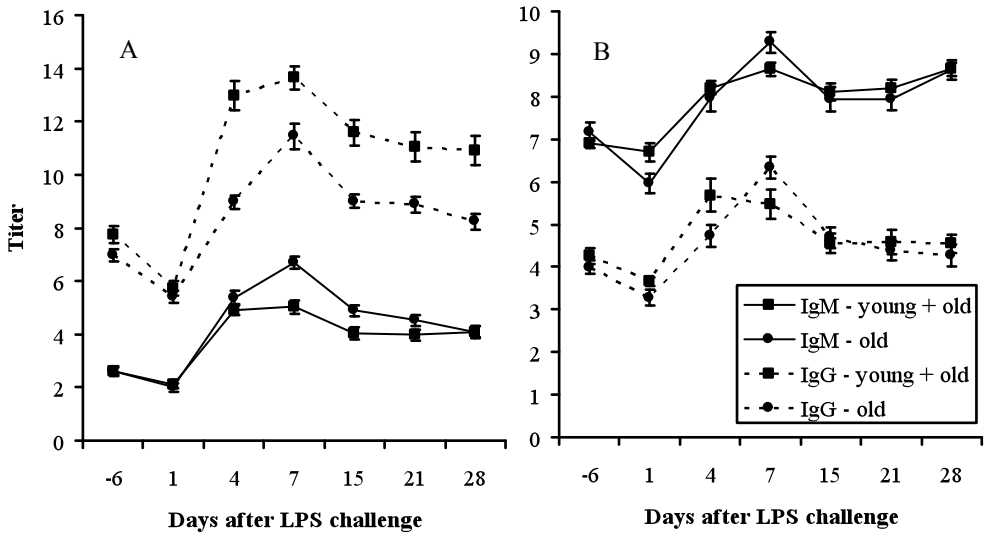

FIGURE 8.7. Kinetics and magnitude of antibodies specified by isotype IgM and IgG for laying hens with early-life LPS experience (young + old) or without LPS experience (old), [A] level of IgM and IgG to lipopolysaccharide. [B] Level of IgM and IgG to keyhole limpet haemocyanin.

environment, where low responsiveness is in favor of robustness. Survival rate, production level, and sensitivity to environmental fluctuations depend on genetic background and type of environmental stressor, and to a minor extent on early-life experiences. Our data suggest that robustness mainly depends on the capacity to respond to stressors within a genetic background, and that the maintenance of different fitness strategies within a selected purebred may favor coping with different environments on the long term.

ROBUSTNESS ON INDIVIDUAL LEVEL

The conclusions about robustness as discussed above were, however, based on line difference. For the final goal of the project, it was important to find traits on individual level, that could be implemented into a breeding goal. Therefore, it is important to select traits for robustness that can be easily measured on a large scale. Egg production is one of the basic elements for robustness and selection on this trait have been applied since decades. In future livestock systems it is necessary to implement new traits into the breeding goal that are related to animal health and welfare (Chapter 2). Interesting traits, that can be measured on a large scale, are performance parameters (feed intake, body

Table 8.4. Principal Component Analysis (PCA) output for the data concerning performance, immune, and physiological parameters of 320 laying hens (data were corrected for line, treatment, and time effects)

PCA variable	Loadings on the five components generated by the PCA				
	PC 1	PC 2	PC 3	PC 4	PC 5
Feed intake	-0.797	0.051	0.081	0.158	-0.154
Body weight	-0.651	0.118	0.230	-0.147	0.197
Hen-day egg production	-0.507	-0.047	-0.184	0.327	-0.549
Egg weight	-0.495	0.044	0.259	-0.053	0.413
NAb to KLH	-0.144	0.493	-0.571	-0.021	0.088
Ab to LPS	-0.208	0.510	-0.557	-0.104	0.120
SpAb to HuSA	0.048	0.117	-0.341	-0.216	0.282
CPW	0.172	0.728	0.341	-0.097	-0.208
APW	0.190	0.689	0.416	0.016	-0.211
Corticosterone	0.093	-0.079	-0.306	-0.313	-0.210
T3	0.081	0.118	0.092	0.328	0.516
Glucose	0.009	0.004	-0.171	0.375	-0.117
Uric acid	-0.280	-0.083	0.166	-0.497	-0.003
TBARS	-0.048	-0.114	0.002	-0.609	-0.198
Variance explained (%)	12.78	11.30	9.72	8.49	7.76

Abbreviation key: PC = principal component; NAb = natural antibody; Ab = antibody; SpAb = specific antibody; KLH = keyhole limpet haemocyanin; LPS = lipopolysaccharide; HuSA = human serum albumin; CPW = classical complement pathway; APW = alternative complement pathway; T3 = 3,5,3'-triiodothyronine; TBARS = thiobarbituric acid reacting substances.

weight, egg weight), but also blood parameters reveal a broad diversity of traits that might be related to health and welfare. The results of these parameters per line are described in Chapter 3 to 7. In this paragraph, data as described in Chapter 4 to 6 will be evaluated by a Principal Component Analysis (PCA), which will give insight in the coherence of parameters on individual level.

The objective of the PCA analysis is to find a small number of factors (the 'principal components') which are linear combinations of the original variables, and which best explain the total variation between animals (Minozzi et al., 2008). The results of the PCA

analysis are shown in Table 8.4 and Figure 8.8. Survival, as basic element of robustness, was not included in the PCA because of the low mortality rate in this experiment.

The first five principal components (PC) explained 50% of the total variance. The variation between individuals in the first principal component (PC 1) is best explained by the performance parameters: feed intake, body weight, hen-day egg production, and egg weight. The variation between individuals in the second principal component (PC 2) is best explained by the innate immune parameters: NAb binding to KLH, Ab binding to LPS, and activity of the classical and alternative complement pathways. The first and second principal components explained 12.8% and 11.3% of the total variance, respectively.

The PCA confirms findings of the former paragraph, that corticosterone is of minor importance for performance and immune parameters in reaction to the environmental stressors. In the PCA, corticosterone does not explain a lot of variation in each of the five principal components.

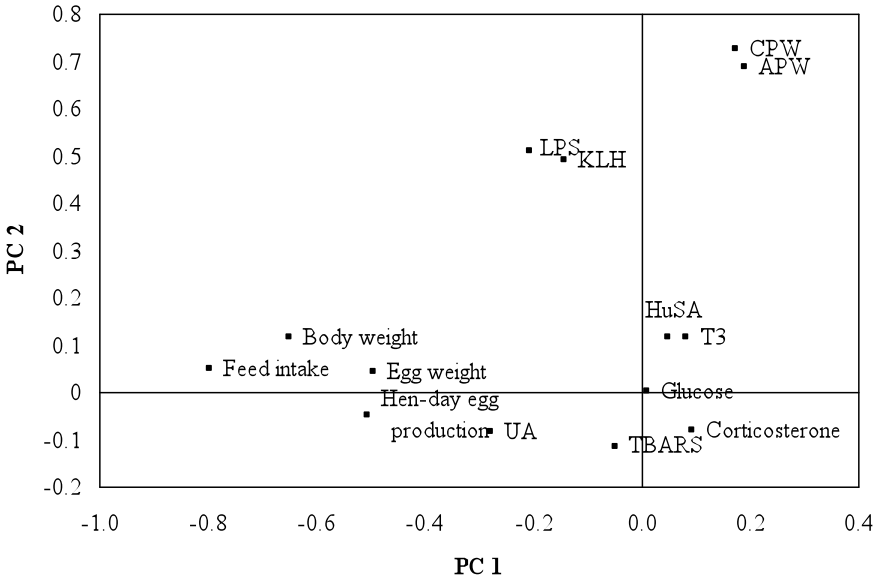

FIGURE 8.8. Output of the Principal Component Analysis (PCA; measurements from 320 laying hens, data were corrected for line, treatment, and time effects). Principal component 1 (PC 1) and principal component 2 (PC 2) explain 12.78% and 11.30% of the variance, respectively. Abbreviation key: KLH = keyhole limpet haemocyanin; LPS = lipopolysaccharide; HuSA = human serum albumin; CPW = classical complement pathway; APW = alternative complement pathway; T3 = 3,5,3'-triiodothyronine; UA = uric acid; TBARS = thiobarbituric acid reacting substances.

The PCA gives an impression on the coherence of parameters on individual level, where performance parameters explain most variation in the first principal component and the immune parameters explain most variation in the second principal component, indicating that performance parameters and innate immune parameters are most likely not related (although the parameters are not totally independent). To confirm this, a Pearson product-moment correlation analysis was performed. This analysis showed that each of the correlation coefficients between performance and innate immune parameters was smaller than 0.10. This indicates that selection on innate immune parameters will probably not be on the expense of performance parameters, and most important not on the expense of hen-day egg production.

Selection on innate immunity is of interest, because levels of NAb binding to KLH, regardless of layer line, were predictive for survival (Chapter 3). Recently, the same relation between level of NAb binding to KLH and mortality rate was established in a crossbred population (W1 × WB; Figure 8.9). An important comment, however, is that the range in titer was smaller than in purebred layer lines (Chapter 3) and that the level of mortality caused by cannibalism is probably high. This last comment implies that the relation between level of NAb binding to KLH and survival is not straightforward, and might be related to abnormal behaviors as cannibalism.

As also described in Chapter 3, level of NAb binding to LPS was also predictive for survival, where higher levels of NAb binding LPS were in favor of survival. In the crossbred population, however, higher levels of NAb binding LPS were not in favor of survival (Figure 8.9). That findings in level of NAb binding to LPS in relation to survival are not consist may have to do with, 1) LPS is common in the environment of the chicken, which makes exposure to LPS an 'unknown factor', 2) chickens were analyzed for NAb binding to *Escherichia coli* LPS, there are, however, many different types of LPS, and together with the fact that LPS is an 'unknown factor' in the environmental, outcomes can differ, 3) related to this, levels of (N)Ab binding to LPS depends on dose (Gehad et al., 2002), and dose is also an 'unknown factor', or 4) high level of cannibalism in the crossbred population.

Besides the relation between NAb and survival, a moderate heritability was estimated for NAb. In the crossbred population (W1 × WB) we have estimated a heritability of NAb binding to KLH of 0.23 and a heritability of 0.17 for NAb binding to LPS. The estimated genetic correlation between NAb binding to KLH and binding to LPS was 0.81 in the crossbred population. This confirms the findings by Siwek et al. (2006), who estimated a heritability of NAb binding to LPS of 0.17 and 0.09 at 5 and 18 weeks of age, respectively,

and a heritability of specific Ab binding to KLH of 0.07 at 12 wk of age in a chicken population selected for primary antibody response to sheep red blood cells. Furthermore, they estimated a heritability of NAb binding to LPS of 0.23 at 38 weeks of age and a heritability of specific Ab to KLH of 0.11 at 36 weeks of age in a chicken population selected for production traits (FP population). Genetic correlation between NAb binding to LPS and specific Ab binding to KLH in the FP population was 0.79.

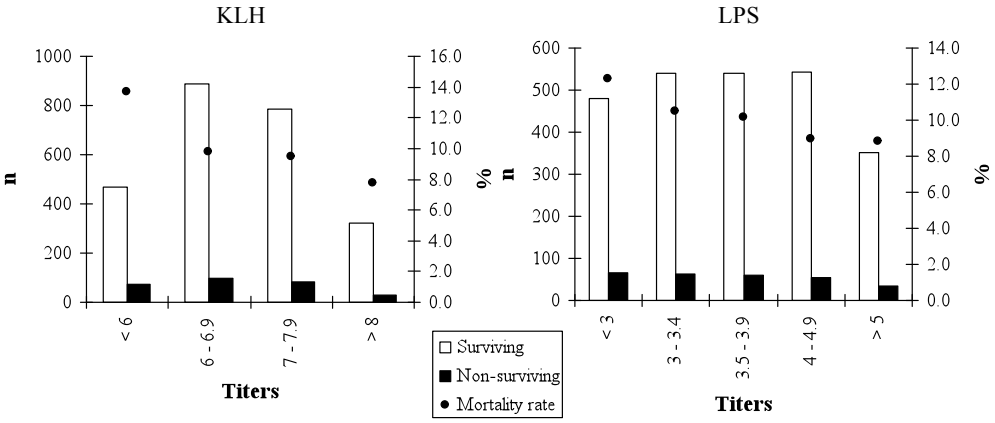

FIGURE 8.9. Mortality rate (%) and number (n) of surviving and non-surviving laying hens classified by levels of natural antibodies (log_2 value) binding to keyhole limpet haemocyanin (KLH) or lipopolysaccharide (LPS).

Besides the relation between NAb and survival, a moderate heritability was estimated for NAb. In the crossbred population (W1 × WB) we have estimated a heritability of NAb binding to KLH of 0.23 and a heritability of 0.17 for NAb binding to LPS. The estimated genetic correlation between NAb binding to KLH and binding to LPS was 0.81 in the crossbred population. This confirms the findings by Siwek et al. (2006), who estimated a heritability of NAb binding to LPS of 0.17 and 0.09 at 5 and 18 weeks of age, respectively, and a heritability of specific Ab binding to KLH of 0.07 at 12 wk of age in a chicken population selected for primary antibody response to sheep red blood cells. Furthermore, they estimated a heritability of NAb binding to LPS of 0.23 at 38 weeks of age and a heritability of specific Ab to KLH of 0.11 at 36 weeks of age in a chicken population selected for production traits (FP population). Genetic correlation between NAb binding to LPS and specific Ab binding to KLH in the FP population was 0.79.

The positive genetic correlation between KLH and LPS as estimated in our experiment and by Siwek et al. (2006), indicates that selection for KLH results in selection for LPS. In

addition, it is more consistent when selection is based on NAb binding to KLH. Levels of (N)Ab binding to LPS depends on dose, LPS is common in the environment of chickens ('unknown factor'), and the relation between survival and LPS levels is not consistent.

Summarizing, in future livestock systems it is necessary that breeding goals should not only be defined in terms of production, but that they should also include traits related to animal health and welfare. Therefore, the findings described in Chapter 3 are of interest and probably most important for robustness. Besides, heritabilities for NAb are moderate, indicating that selection for NAb levels is possible. In practical - commercial - context, however, selection for this 'robustness' trait must be in balance with selection for production traits, which is probably the case, as indicated by the PCA analysis.

CONCLUSION

The aim of the project 'The genetics of robustness in laying hens' was to investigate nature and regulation of robustness in laying hens under sub-optimal conditions and the possibility to increase robustness by using animal breeding without loss of production.

Robustness is a property that allows an organism to maintain its functions despite external and internal influences with the ultimate goal to survive and reproduce. Based on this, the basic elements for robustness in biology, but also in animal production, are survival and reproduction. Besides, a robust animal has to respond to unpredictable fluctuations in the environment, where a robust animal has a low responsiveness towards environmental stressors enabling the animal to maintain body functions within limits. Concisely, a robust laying hen is a hen with a high survival rate, high production level, and low responsiveness towards environmental stressors.

Our results indicate that robust layer lines do already exist, where some lines are more robust than others depending on genetic background and type of environmental stressor. From the lines evaluated in this thesis, line WF can be characterized as a robust layer line, because this line had a high survival rate, high production level, and was low responsive towards environmental stressors.

For the final goal of the project, it was important to find traits on individual level that could be implemented into a breeding goal. Poultry breeding already takes advantage of the basic robustness elements, e.g., survival and reproduction. A potential key factor influencing these basic robustness elements might be defense by the immune system to invading pathogens. There is evidence that natural antibodies, as part of the innate immune system, play a major role in survival. We have established a predictive value for the level of NAb binding to keyhole limpet haemocyanin for survival of the laying period by laying

hens. Furthermore, NAb have a moderate heritability, showing opportunity for selection towards this trait. Performance parameters and innate immune parameters are most likely not related and selection on innate immune parameters will probably not be on the expense of hen-day egg production. Implementation of selection for NAb into a breeding goal might, therefore, improve robustness of laying hens.

REMARKS AND RECOMMENDATIONS

The research described in this thesis is mainly based on data measured in purebred layer lines. To get a better understanding of robustness and the influence of genetic background, the use of purebred lines was well chosen. The robust laying hen that the breeding company has in mind is a chicken for commercial purposes, and this is always a crossbred chicken. To breed a robust chicken it is therefore recommendable to use crossbred layer lines.

The same can be recommended for type of housing system. In this thesis, laying hens were always housed in cages, individually or with 4 to 5 hens per cage. Worldwide, most laying hens are kept in cages, but for instance in Western Europe, housing of laying hens in cages will become forbidden. Floor housing, free range, and organic systems are becoming established in this part of the world. The breeding company should orientate towards housing systems other than cages, because a robust laying hen in a cage system might not be a robust laying hen in another housing system.

How laying hens respond to environmental stressors is of interest for robustness. In this thesis it is described that a low responsiveness towards environmental stressors is in favor of robustness. Further investigation of this phenomenon is recommendable, because it seems to depend on genotype and type of environmental stressor, and can be very descriptive for robustness and the traits that are most related to robustness.

The results of the experiments described in this thesis all focused on differences between layer lines. In the final Chapter, robustness is evaluated on individual level. More effort has to be made for evaluation of robustness on individual level. This is the only way to determine interesting traits that can be implemented into a breeding goal for robustness.

An interesting trait that can be implemented into a breeding goal for robustness is level of natural antibodies. Level of natural antibodies seems to be predictive for survival. The function of NAb, however, is unknown and it is unclear yet if the relation between NAb and survival depends on level of NAb or expression of NAb. Further investigation of this relation is also necessary, because the results are not always consistent yet. In addition, this inconsistency might be related to abnormal behavior, cannibalism, which is also a major

problem in poultry farming. It is worthwhile to investigate the relation between levels or expression of NAb and the expression of abnormal behavior.

The ultimate goal of the project 'The genetics of robustness in laying hens' was to implement traits of interest for robustness into a breeding goal. There is evidence that the level of NAb is of interest for robustness, and besides, differences in genetic background with regard to NAb exist and are moderately heritable. This gives opportunity for selection towards this trait. Implementation of NAb into a breeding goal for robustness has to be realized.

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Summary

Introduction

Aim of the project 'The genetics of robustness in laying hens' was to investigate nature and regulation of robustness in laying hens under sub-optimal conditions and the possibility to increase robustness by using animal breeding without loss of production. At the start of the project, a robust animal was defined as 'an animal under a normal physical condition that has the potential to keep functioning and take short periods to recover under varying environmental conditions'. Next, parameters or traits were selected that could give an indication of robustness, and that could be implemented into a breeding goal for robustness. The experiments described in this thesis investigated parameters of interest for robustness in laying hens, where the influence of genetic background, environmental conditions, and early-life experiences was used as framework.

Genetic background

Aim of the first experiment (Chapter 3) was to establish genetic differences in innate (natural) humoral immune components between 12 purebred layer lines and the relation between natural antibodies (NAb) and survival. Six White Leghorn lines (W1, WA, WB, WC, WD, WF) and 6 Rhode Island Red lines (B1, B2, B3, BA, BB, BE), housed in the same facility with 4 hens per cage, were followed during one laying period. At 20, 40, and 65 weeks of age, blood samples were collected. Serum was used to study innate immune competence of laying hens by measuring levels of NAb binding to keyhole limpet haemocyanin (KLH) or lipopolysaccharide (LPS), respectively, and haemolytic (classical and alternative) complement activity.

A distinction could be made between lines showing high or low immune competence with respect to NAb and complement activity. Within lines, significant correlations were found for each of the innate immune parameters among the three ages. Innate immune parameters were, however, not correlated with each other. Based on the limited data set, it was not possible to draw conclusions on line differences for innate immune competence in relation to survival. However, regardless of line, low levels of NAb binding to KLH or high levels of NAb binding to LPS were detected in hens that did not survive the laying period. The major difference between responses of NAb binding to KLH or LPS was that hens probably did not encounter KLH, which suggests a reflection of the capacity to respond, whereas hens most probably did encounter LPS, which suggests a reflection of the active status of the innate humoral immune system. This study showed that levels (KLH) and

activation (LPS) of components of natural antibodies are indicative for the probability that hens survive a laying period.

ENVIRONMENTAL INFLUENCE

Aim of the second experiment (Chapter 4 to 6) was to establish the influence of, or response to, environmental stressors. From the former experiment, 4 of the 12 lines (WA, WB, WF, and B1) were selected, based on a profile of high or low innate immune competence and high or low survival rate. Hens of these lines were exposed to the following environmental stressors: heat (climatic stress), LPS (hygienic stress), or combined exposure to heat and LPS. At 22 weeks of age, 80 hens per line were randomly divided over 2 identical climate chambers, and, at 24 weeks of age, exposed to a constant high temperature (32ºC) or control temperature (21ºC) for 23 days. Half of the hens housed in each chamber were i.v. injected with 1 mg/kg of BW of LPS at day 1 after the start of heat stress. Effect of heat, LPS, or combined challenge on immune competence (Chapter 4), performance (Chapter 5), and physiological responses (Chapter 6) were investigated.

It was hypothesized that laying hens would be able to cope with single environmental stressors, but that problems in coping ability would occur when hens were exposed to combined environmental stressors. In general, however, hens were able to cope with single heat and LPS challenge, but were also able to cope with combined exposure to heat and LPS. Lipopolysaccharide and heat stress initiated sequential responses over time, with an earlier effect of short-term LPS exposure (within the first and second week) and a later effect of long-term heat exposure (within the second and third week), indicating that heat stress and LPS challenge acted like two independent stressors. The layer lines had similar response patterns, but differed in response levels, suggesting that some lines were better able to adapt to environmental stressors than others. Furthermore, neither innate immune competence nor survival rate, on which bases the lines were characterized, was indicative for the response to different stressors.

EARLY-LIFE EXPERIENCE

Aim of the third experiment (Chapter 7) was to improve adaptability to heat stress or LPS challenge by conditioning the laying hens, preferably during early-life, to prepare them for unexpected heat spells or changes in hygienic circumstances. Line B1 was chosen for this experiment, mainly because this line was not able to maintain a high hen-day egg production during heat stress. Chicks were exposed to 37ºC for 24 hours at day 5 of age, or were i.v. injected once with 1 mg/kg of BW of LPS at 6 weeks of age, or were exposed to

both stressors, while a control group was reared under standard conditions receiving a placebo treatment of PBS. At 24 weeks of age, hens treated in early-life were re-exposed to a similar stressor. Early-life control hens were exposed to heat stress, i.v. injected with LPS, or exposed to both stressors.

Early-life heat stress experience and early-life LPS experience did not affect adaptability of these laying hens towards heat stress or LPS challenge in later-life, e.g., no difference in feed intake, BW, hen-day egg production, and egg weight. Early-life LPS experience did affect, however, kinetics and magnitude of (N)Ab levels binding to LPS and KLH, indicating that early-life LPS exposure can enhance the status of immune reactivity or induce a higher sensitivity to LPS in later-life.

Robustness: survival, production, and flexibility

Based on the results of these experiments, it can be concluded that robustness mainly depends on the capacity to respond to stressors within a genetic background, and that the maintenance of different fitness strategies within a selected purebred line may favor coping with different environments on the long term. Next, it is important to determine parameters or traits that are of interest for robustness. From a biological point-of view, but also from a poultry production point-of-view, the basic elements for robustness are survival and reproduction, and these specific functions have to be maintained. Furthermore, robustness can be indicated by sensitivity to unpredictable fluctuations in the environment, where low responsiveness is in favor of robustness.

Breeding for robustness

Survival rate, production level, and sensitivity to environmental fluctuations depend on genetic background and type of environmental stressor, and to a minor extent on early-life experiences. These results and statements about robustness are, however, based on line differences. For the final goal of the project, it was important to find traits on individual level that could be implemented into a breeding goal. Therefore, it is important to select traits for robustness that are predictive and can be easily measured on a large scale. Egg production is one of the basic elements for robustness and selection on this trait have been applied since decades. Furthermore, it was shown that there is a relation between level of NAb and the probability to survive, and this finding is of interest for robustness. Besides, heritabilities for NAb are moderate, indicating that selection for NAb levels is possible and could be implemented into a breeding goal for robustness.

Ethical aspects

Improving robustness by selective breeding will increase (or restore) the ability of animals to interact successfully with the environment and thereby to make them more able to adapt to an appropriate husbandry system (Chapter 2). In future livestock systems it is necessary that breeding goals should not only be defined in terms of reproduction, but that they also include traits related to animal health and welfare. Interesting traits, that can be measured on a large scale, are performance parameters (feed intake, body weight, egg weight), but also blood parameters reveal a broad diversity of traits that might be related to health and welfare. In practical - commercial - context, selection for these robustness traits must be in balance with selection for production traits. For a successful implementation of robustness as a breeding goal, it is, however, important to implement all traits, because the success of selective breeding for robustness depends on all traits and not on a singular trait. Besides, in the future other traits may arise that have to be implemented into the breeding goal of robustness. By implementation of new traits, it is, however, important that these traits concern the animal itself.

Conclusion

The basic elements for robustness are survival, reproduction, and responsiveness towards environmental stressors, where a robust laying hen is a hen with a high survival rate, high production level, and low responsiveness towards environmental stressors. We have established a predictive value for the level of NAb binding to KLH for survival of the laying period of laying hens. Besides, NAb have a moderate heritability, giving opportunity for selection towards this trait. Performance parameters and innate immune parameters are most likely not related and selection on innate immune parameters will probably not be on the expense of hen-day egg production. Implementation of selection for NAb into a breeding goal might, therefore, improve robustness of laying hens.

Samenvatting

Introductie

Het doel van het project 'De genetica van robuustheid in leghennen' was om de regulatie van robuustheid van leghennen onder suboptimale omstandigheden te bestuderen en mogelijkheden te inventariseren om robuustheid te vergroten door middel van fokkerij zonder dat dit ten koste gaat van productie. Aan het begin van het project werd een robuust dier gedefinieerd als 'een dier onder normale fysieke omstandigheden met potentie om te blijven functioneren en snel te herstellen onder wisselende omgevingsinvloeden'. Vervolgens werden parameters of kenmerken geselecteerd die een indicatie zouden kunnen geven over robuustheid, om geïmplementeerd te kunnen worden in een fokdoel voor robuustheid. De experimenten beschreven in dit proefschrift bestuderen parameters die interessant zijn voor robuustheid van leghennen, waarbij de invloed van genetische achtergrond, omgevingsinvloeden, en ervaringen op jonge leeftijd als kapstok wordt gebruikt.

Genetische achtergrond

Het eerste experiment (Hoofdstuk 3) had als doel genetische verschillen in innate humorale immuun componenten tussen 12 zuivere leghenlijnen aan te tonen en om de relatie tussen natuurlijke antilichamen (NAb) en overleving te bestuderen. Zes White Leghorn lijnen (W1, WA, WB, WC, WD, WF) en 6 Rhode Island Red lijnen (B1, B2, B3, BA, BB, BE), gehuisvest in dezelfde stal met 4 hennen per kooi, zijn bestudeerd gedurende een legperiode. Op 20, 40, en 65 weken leeftijd zijn bloedmonsters genomen. Serum werd gebruikt voor het bepalen van innate immuun competentie in leghennen door bepaling van het niveau aan NAb tegen keyhole limpet haemocyanin (KLH) en lipopolysaccharide (LPS), respectievelijk, en (klassieke en alternatieve) complement activiteit.

Op basis van NAb en complement activiteit kan onderscheidt gemaakt worden tussen lijnen met een hoge of lage innate immuun competentie. Binnen lijnen werden significante correlaties gevonden tussen de drie verschillende leeftijden voor elk van de innate immuun parameters. De innate immuun parameters waren echter niet met elkaar gecorreleerd. Door de kleine dataset was het niet mogelijk om lijnverschillen vast te stellen voor de relatie tussen innate immuun competentie en overleving. Onafhankelijk van lijn kon wel worden aangetoond dat hennen met een laag niveau aan NAb tegen KLH of een hoog niveau aan NAb tegen LPS een grotere kans hebben om de legperiode niet te overleven. Het verschil tussen NAb tegen KLH of LPS is dat hennen nooit in aanraking zijn geweest met KLH, waardoor KLH de capaciteit om te reageren reflecteert, maar waarschijnlijk wel in

aanraking zijn geweest met LPS, waardoor LPS de actieve status van het innate immuun systeem reflecteert. Deze studie laat zien dat het niveau (KLH) en de activiteit (LPS) van componenten van NAb voorspellend zijn voor de kans dat hennen de legperiode overleven.

OMGEVINGSINVLOEDEN

Het tweede experiment (Hoofdstuk 4 tot 6) had als doel de invloed van omgevings-stressoren vast te stellen. Van het vorige experiment werden 4 van de 12 lijnen (WA, WB, WF, B1) geselecteerd op basis van hoge of lage innate immuun competentie en een hoog of laag overlevingspercentage. De lijnen werden blootgesteld aan de volgende omgevings-stressoren: hitte (klimaat stress), LPS (hygiëne stress), of aan beide stressoren. Op 22 weken leeftijd werden 80 hennen per lijn verdeeld over 2 identieke klimaatkamers. Op 24 weken leeftijd werden ze blootgesteld aan een constante hoge temperatuur (32ºC) of een controle temperatuur (21º) gedurende 23 dagen. Een dag na aanvang van de hitte stress werd in elke kamer de helft van de hennen i.v. geïnjecteerd met 1 mg/kg lichaamsgewicht aan LPS. Het effect van hitte, LPS, of gecombineerd blootstelling op immuun competentie (Hoofdstuk 4), prestatie (Hoofdstuk 5), en fysiologische reacties (Hoofdstuk 6) werden bestudeerd.

De verwachting was dat hennen in staat zouden zijn zich aan te passen aan een enkele omgevingsstressor, maar dat aanpassingsproblemen op zouden treden als hennen blootgesteld worden aan gecombineerde stressoren. In het algemeen waren hennen in staat zich aan te passen aan hitte of LPS, maar ze waren ook instaat zich aan te passen bij blootstelling aan de combinatie van hitte en LPS. Lipopolysaccharide en hitte stress initieerde achtereen-volgende reacties over tijd, met een vroeg effect van de kortdurende blootstelling aan LPS (in de eerste en tweede week) en een later effect van de langdurende blootstelling aan hitte (in de tweede en derde week), wat aantoont dat hitte en LPS zich gedragen als twee onafhankelijke stressoren. De leghenlijnen hadden een vergelijkbaar reactiepatroon, maar verschilde in reactieniveau, wat suggereert dat sommige lijnen beter in staat zijn zich aan te passen aan omgevingsstressoren dan andere. Verder waren de lijnen geselecteerd op basis van innate immuun competentie en overlevingspercentage, maar beide lijken niet voorspellend voor de reactie op de verschillende stressoren.

ERVARING OPGEDAAN OP JONGE LEEFTIJD

Het derde experiment (Hoofdstuk 7) had als doel het adaptatievermogen voor hitte en LPS te verbeteren door leghennen te conditioneren, bij voorkeur op jonge leeftijd, om ze voor te bereiden op onverwachte hitte periodes en veranderingen in hygiënische omstandigheden.

Voor dit experiment werd lijn B1 geselecteerd, omdat deze lijn minder in staat was een hoge productie te behouden tijdens hitte stress. Kuikens werden op 5 dagen leeftijd blootgesteld aan 37ºC gedurende 24 uur, of werden op 6 weken leeftijd eenmalig i.v. geïnjecteerd met 1 mg/kg lichaamsgewicht aan LPS, of werden blootgesteld aan beide stressoren. Een controle groep werd onder standaard condities gehouden en geïnjecteerd met een placebo. Op 24 weken leeftijd werden hennen met ervaring op jonge leeftijd opnieuw blootgesteld aan dezelfde soort stressor. Controle hennen werden blootgesteld aan hitte, i.v. geïnjecteerd met LPS, of blootgesteld aan beide stressoren.

Blootstelling aan hitte of LPS op jonge leeftijd verbeterde het adaptatievermogen van leghennen op blootstelling aan dezelfde stressoren op later leeftijd niet; geen verschil in voeropname, lichaamsgewicht, productie, en ei gewicht. Blootstelling aan LPS op jonge leeftijd had echter invloed op het niveau en het patroon aan antilichamen tegen LPS en KLH, wat aangeeft dat blootstelling aan LPS op jonge leeftijd de immuun reactiviteit op latere leeftijd kan verhogen of een hogere gevoeligheid voor LPS kan veroorzaken.

Robuustheid: overleving, productie, en flexibiliteit

Gebaseerd op bovenstaande experimenten, kan geconcludeerd worden dat robuustheid afhangt van de mogelijkheid om te reageren op een stressor gegeven de genetische achtergrond, en dat bepaalde fitness strategieën op de lange termijn gunstig zijn voor aanpassing van lijnen aan verschillende omgevingen. Daarnaast is het van belang om parameters of kenmerken vast te stellen die interessant zijn voor robuustheid. Reproductie en overleving zijn hierbij de basis elementen voor robuustheid, en deze specifieke functies moeten in stand gehouden worden. Daarnaast kan robuustheid bepaald worden aan de gevoeligheid voor onvoorspelbare fluctuaties in de omgeving, waarbij een lage gevoeligheid gunstig is voor robuustheid.

Selecteren voor robuustheid

Overlevingspercentage, productie niveau, en gevoeligheid voor fluctuaties in de omgeving zijn afhankelijk van genetisch achtergrond en aard van de omgevingsstressor, en in mindere mate van ervaring opgedaan op jonge leeftijd. Deze resultaten en uitspraken over robuustheid zijn echter gebaseerd op lijnverschillen. Voor het uiteindelijke doel van het project is het noodzakelijk om individuele kenmerken voor robuustheid vast te stellen zodat deze geïmplementeerd kunnen worden in een fokdoel. Het is daarom van belang om kenmerken te selecteren die voorspellend zijn voor robuustheid en die gemakkelijk op grootte schaal te meten zijn. Eiproductie is een van de basis elementen voor robuustheid en

selectie voor dit kenmerk wordt al sinds decennia toegepast. Verder is een relatie aangetoond tussen het niveau aan NAb en kans om te overleven, en deze bevinding is interessant voor robuustheid, met name vanwege de gemiddelde hoogte van de geschatte erfelijkheidsgraad voor NAb, wat aangeeft dat selectie voor NAb niveau mogelijk is en geïmplementeerd zou kunnen worden in een fokdoel voor robuustheid.

ETHISCHE ASPECTEN

Het verbeteren van robuustheid door middel van fokkerij zal dieren beter in staat stellen om te gaan met de omgeving en daardoor zullen ze zich beter aan kunnen passen aan een geschikt huisvestingssysteem (Hoofdstuk 2). In toekomstige dierlijke productiesystemen is het van belang dat fokdoelen niet alleen gericht zijn op (re)productie, maar dat ook rekening wordt gehouden met diergezondheid en welzijn. Interessante kenmerken, die gemakkelijk op grootte schaal te meten zijn, zijn prestatieparameters (voeropname, lichaamsgewicht, ei gewicht), maar ook bloedparameters kunnen een breed scala aan kenmerken aan het licht brengen die gerelateerd zijn aan gezondheid en welzijn. In de praktijk - commercieel - mag selectie op deze robuustheidskenmerken niet ten koste gaan van productiekenmerken. Voor een succesvolle toepassing van robuustheid in een fokdoel is het echter van belang om meerdere kenmerken te implementeren, want het succes van het fokken van robuuste dieren hangt af van meerdere kenmerken en niet van een enkel kenmerk. Verder zullen in de toekomst andere kenmerken naar voren komen die van belang zijn voor robuustheid. Bij het implementeren van nieuwe kenmerken in het fokdoel is het echter belangrijk dat deze kenmerken van belang zijn voor het dier zelf.

CONCLUSIE

De basis elementen voor robuustheid zijn overleving, reproductie, en gevoeligheid voor omgevingsstressoren, waarbij een robuuste leghen wordt gezien als een hen met een hoog overlevingspercentage, hoog productieniveau, en een lage gevoeligheid voor omgevings-stressoren. Dit onderzoek toont aan dat het niveau aan NAb tegen KLH voorspellend is voor het overleven van de legperiode door hennen. Daarnaast is de geschatte erfelijkheidsgraad voor NAb gemiddeld, wat mogelijkheden biedt voor selectie op dit kenmerk. Prestatieparameters en innate immuun parameters zijn waarschijnlijk niet gerelateerd en selectie op innate immuun parameters gaat waarschijnlijk niet ten kosten van eiproductie. Implementatie en selectie van NAb voor een fokdoel kan daarom de robuustheid van leghennen verbeteren.

Curriculum vitae

CURRICULUM VITAE (Nederlands)

Laura Star werd geboren op 6 december 1979 in Leiderdorp en groeide op in Lelystad. In 1998 behaalde zij haar VWO diploma aan Scholengemeenschap De Rietlanden te Lelystad. Datzelfde jaar begon zij met de opleiding Zoötechniek aan Wageningen Universiteit & Research. In november 2003 werd de MSc Animal Science afgerond met afstudeervakken bij Gezondheidsleer & Reproductie en Product Design & Quality, en stage bij Praktijkonderzoek Veehouderij te Lelystad en aan Massey University, Palmerston North, Nieuw Zeeland. In mei 2004 begon zij als assistent in opleiding (AIO) bij de leerstoelgroep Adaptatiefysiologie van Wageningen Universiteit & Research. Het daar uitgevoerde onderzoek staat beschreven in dit proefschrift. Sinds juni 2008 werkt zij als onderzoeker bij Schothorst Feed Research B.V. te Lelystad.

CURRICULUM VITAE (English)

Laura Star was born on December 6th 1979 in Leiderdorp and was raised in Lelystad. In 1998, she graduated from high school Scholengemeenschap De Rietlanden in Lelystad. During the same year, she started with the BSc Animal Science at Wageningen University & Research. In November of 2003, she completed her MSc with theses on Health & Reproduction and Product, Design & Quality, and internship at Praktijkonderzoek Veehouderij in Lelystad and at Massey University in New Zealand. In May 2004 she started as a PhD student at the Adaptation Physiology Group of Wageningen University & Research. The research performed there is described in this thesis. Since June 2008 she is employed as researcher at Schothorst Feed Research B.V. in Lelystad, the Netherlands.

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Training and supervision plan

The basic package (3 ects)

WIAS introduction course	2005
Course on philosophy of science and ethics	2005

International conferences (15 ects)

NE-1016: Genetic Bases for Resistance and Immunity to Avian Diseases, Clemson, USA	2005
NE-1016: Genetic Bases for Resistance and Immunity to Avian Diseases, Davis, USA	2006
NE-1016: Genetic Bases for Resistance and Immunity to Avian Diseases, Guelph, Canada	2007
Benelux Congress of Zoology, Wageningen, the Netherlands	2005
EurSAFE: Ethics and the politics of Food, Oslo, Norway	2006
Avian Immunology Research Group, Paris, France	2006
Avian Immunology Research Group, Queensland, Australia	2008
World's Poultry Congress, Brisbane, Australia	2008

Seminars and workshops (6 ects)

WIAS Science Day, Wageningen, the Netherlands	2005-2008
PhD retreat, Nijmegen, the Netherlands	2004
Symposium 'Compartmentalization of the immune response', Lunteren, the Netherlands	2007
WIAS Seminar 'Immune response to viruses: a comparable approach', Wageningen, the Netherlands	2006
WIAS Seminar 'Science meets society', Wageningen, the Netherlands	2007
WIAS Seminar 'Personalities in animals', Wageningen, the Netherlands	2007
WIAS Seminar 'Strategies to improve health and fertility in dairy cows', Wageningen, the Netherlands	2008
EADGENE: Local Ethical Matrix Workshop; Wageningen, the Netherlands	2008

In-depth studies (9 ects)

Advances in quantitative genetics	2004
RNAi technique and application in viral disease in plant and animals	2004
Advanced statistics course: experimental design	2005
Science meets society: robustness in the context of animal production	2006
Debating course 'Biology underpinning animal sciences: broaden your horizon'	2007
ELISA: basic understanding and trouble shooting	2007
Mathematical modelling in biology	2008

Professional skills support courses (6 ects)

Supervising MSc thesis work	2004
Techniques for scientific writing	2005
Time planning and project management	2006
Writing winning grant proposal	2007
Gespreksvaardigheden (één op één begeleiding)	2007

Research skills training (6 ects)

Preparing own PhD research proposal	2004

Didactic skills training: teaching and supervising (6 ects)

Lecture course Adaptation Physiology 1 (2×)	2007-2008
Practical Adaptation Physiology 1	2008
Supervising 1 MSc student and 1 intern	2004 / 2007
EADGENE: Local Ethical Matrix Workshop	2008

Management skills training (4 ects)

Organization WIAS Science Day (2×)	2007-2008

Education and training total	**55 ects**

Colophon

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