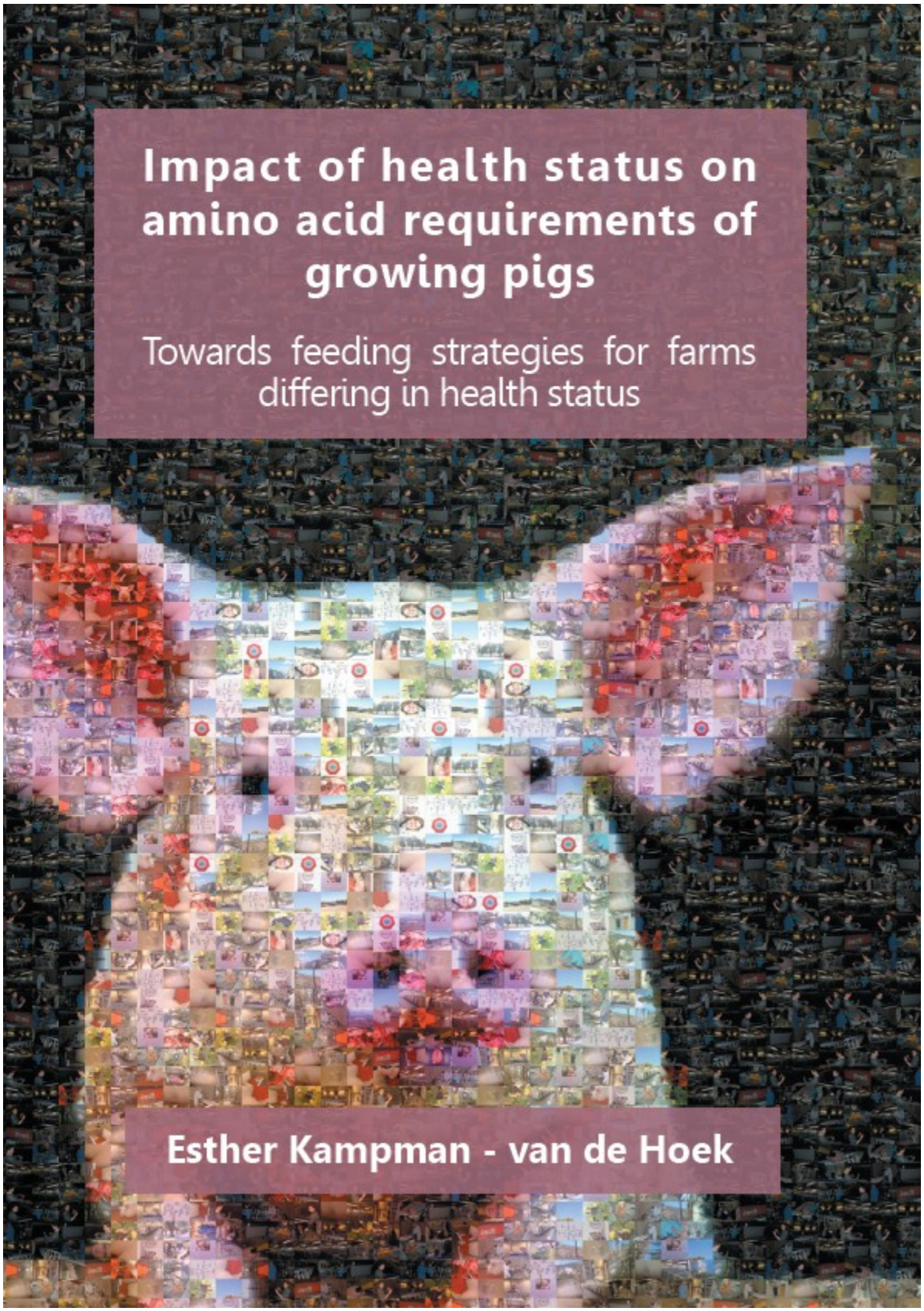

Impact of health status on amino acid requirements of growing pigs
Towards feeding strategies for farms differing in health status
Esther Kampman - van de Hoek

Impact of health status on amino acid requirements of growing pigs

Towards feeding strategies for farms differing in health status

Esther Kampman – van de Hoek

Thesis committee

Promotor
Prof. Dr W.H. Hendriks
Professor of Animal Nutrition
Wageningen University

Co-promotors
Dr W.J.J. Gerrits
Associate professor, Animal Nutrition Group
Wageningen University

Dr A.J.M. Jansman
Senior scientist, Animal Nutrition Group
Wageningen UR Livestock Research

Other members
Prof. Dr D. Eckersall, University of Glasgow, United Kingdom
Dr N. Le Floc'h, INRA, Saint Gilles, France
Prof. Dr J. Keijer, Wageningen University
Prof. Dr M.T. Kidd, University of Arkansas, United States of America

This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS)

Impact of health status on amino acid requirements of growing pigs

Towards feeding strategies for farms differing in health status

Esther Kampman – van de Hoek

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Academic Board,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 12 June 2015
at 11 a.m. in the Aula.

Esther Kampman – van de Hoek

Impact of health status on amino acid requirements of growing pigs.

Towards feeding strategies for farms differing in health status,

184 pages.

PhD thesis, Wageningen University, Wageningen, NL (2015)

With references, with summaries in Dutch and English

ISBN 978-94-6257-343-7

He can who thinks he can

(Orison Swett Marden)

To Frank

Abstract

There is large variation in the production performance of commercial growing-finishing pig farms. This variation even exists when pigs have a similar genetic background and fed similar diets. The health status is one of the major factors contributing to this large variation in pig performance, as activation of the immune system can decrease feed intake, body weight gain and increase nutrient utilisation for immune system functioning. As a consequence, amino acids (AA) are repartitioned from skeletal muscle deposition towards utilisation for immune system functioning. Current requirement estimates for growing-finishing pigs are formulated to maximize protein deposition for growth and do not take into account the increased utilization of AA for immune functioning as induced by health challenging conditions. This lack of knowledge hampers the ability of feed manufacturers to optimize diets and improve pig performance. The main objective of the present thesis was to quantify the effect of health status on AA requirements for body protein deposition and for immune system functioning of growing pigs.

A health status web was developed as a tool to categorize growing-finishing pig farms on the basis of their health status. The health status web can be of use for feed manufacturers to develop targeted strategies to accommodate the nutritional requirements of pigs belonging to particular groups of farms sharing a common health status. A dose-response technique was developed, which is a simple, accurate technique to quantitatively estimate changes in AA requirements of individual meal-fed pigs. Nevertheless, a minimum time period of 21 days is required for each individual, which makes the technique inappropriate for studying the effect of immune system activation on AA requirements. The combined measurements of whole body N retention, plasma irreversible loss rate (ILR, *i.e.* the amount of free AA that disappears per unit of time from the plasma pool for protein synthesis or oxidation), urea entry and appearance of ^{13}C into plasma proteins, provided insight into the consequences of immune system activation on AA metabolism.

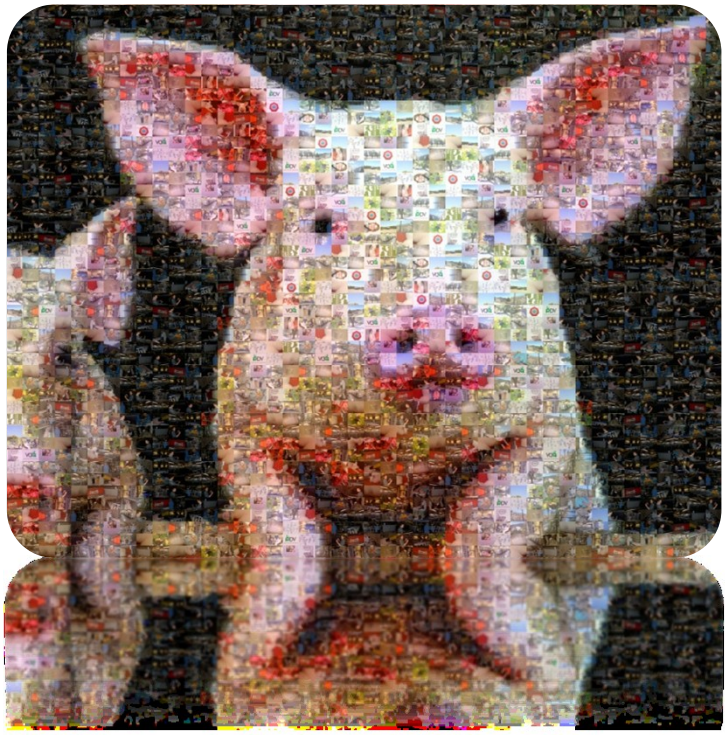
Pigs selected from a farm with a suboptimal health status had greater serum haptoglobin, lower serum albumin concentrations, and greater leukocyte counts in blood at the start of the experiment than pigs selected from a farm with a high health status, indicating a higher level of immune system activation. The occurrence of compensatory gain in pigs from a farm characterized as having a suboptimal health status proves, however, that it is difficult to maintain a contrast in health status, and that pigs can adapt quickly to a change in housing conditions. In the absence of effects on feed intake, health challenging conditions may affect performance due to alterations in post-absorptive AA metabolism, as also indicated by increased urinary N losses, and a tendency for a reduced N retention and a lower utilization of digestible N for N retention in pigs with a systemic inflammation, or by a reduction in faecal nutrient digestibility as indicated for dry matter and N in pigs from a farm with a suboptimal health status. The observed changes in protein and AA metabolism after immune stimulation imply that especially tryptophan may become limiting during immune system activation, whereas lysine becomes excessive. Furthermore, the utilization of methionine, tyrosine, and valine for immune system functioning seems to increase in pigs with a systemic lung inflammation. In addition, the dietary AA or protein supply was able to modulate the acute phase response pre- and post-challenge, stressing the importance of an adequate dietary AA supply for appropriate functioning of the immune system of growing-finishing pigs.

Before implementing targeted feeding strategies for farms sharing a common health status, future research should be conducted to study the possible beneficial effects of increasing the dietary supply of particularly tryptophan, methionine, tyrosine, and valine relative to lysine for immune system function and for body protein deposition in pigs from farms with a different health status.

Table of Contents

Chapter 1

General Introduction



Introduction

Large variation in the performance of growing-finishing pigs exists between commercial pig farms, with average growth rates varying between 570 and 930 g/day based on data from 887 farms in the Netherlands (Figure 1.1) (AgroVision, 2012). The health status of pigs may be an important factor contributing to this large variation in pig performance (Van der Peet-Schwering and Jansman, 2007; Pastorelli *et al.*, 2012). In commercial pig farms the animals can be continuously exposed to (non-)pathogenic agents, which can activate the immune system. During immune system activation, nutrients are redistributed from anabolic and maintenance processes towards processes involved in immunity (Klasing and Johnstone, 1991; Spurlock, 1997; Colditz, 2002). A cascade of cytokine induced metabolic alterations occur, including anorexia, increased breakdown and decreased synthesis of skeletal muscle protein, increased hepatic synthesis of acute phase proteins (APP), and increased deamination of glucogenic amino acids (AA) (Klasing and Johnstone, 1991; Lochmiller and Deerenberg, 2000; Le Floc'h *et al.*, 2004). In pigs, immune system activation decreased feed intake, body weight (BW) gain, and N retention (Williams *et al.*, 1997b; Daiwen *et al.*, 2008; Le Floc'h *et al.*, 2008). An overview of the impact of pathogens or other antigens on protein and AA metabolism is presented in Figure 1.2. Pathogens or antigens activate the immune system and reduce dietary intake and intestinal absorption of AA, and body protein synthesis for growth. Pathogens or antigens, however, stimulate endogenous losses, protein synthesis for immune functioning, protein breakdown, and AA oxidation. As a consequence of a reduction in maximum protein deposition observed after immune system activation, the absolute daily Lys requirements for maximum daily gain and for gain to feed ratio in pigs (Williams *et al.*, 1997c) and to maximize protein deposition in chicken (Webel *et al.*, 1998) can be reduced. However, quantitative information about the effect of immune system activation on the AA requirements of pigs is limited, and measurements on changes in responses of multiple AA to immune system activation are largely absent. In addition, AA requirement studies are often performed in pigs housed in a controlled environment with low pathogen pressure, and do not consider health status as a factor contributing to variation in AA requirements. This lack of knowledge hampers the ability of feed manufacturers to optimize pig diets by adjusting to variation in health status, and thereby to contribute to further improving pig performance. Apart from the influence of immune system activation on AA requirements, there is increasing evidence that the dietary protein or AA supply can affect the inflammatory response during immune system activation (Grimble *et al.*, 1992; Jahoor *et al.*, 1999; Li *et al.*, 1999; Grimble, 2001; Li *et al.*, 2007; Le Floc'h *et al.*, 2008; Le Floc'h *et al.*, 2009; Calder and Yaqoob, 2012). In the present thesis, AA requirements are defined as the optimal dietary AA supply for maximizing body protein deposition and for optimizing immune system functioning. It is hypothesized that feeding diets adjusted to variation in health status improves overall nutrient utilization, leading to improved production performance, and consequently a reduced N excretion into the environment, while maintaining

appropriate functioning of the immune system. Feeding adjusted diets may also support the pig's capacity to cope with challenges to the immune system.

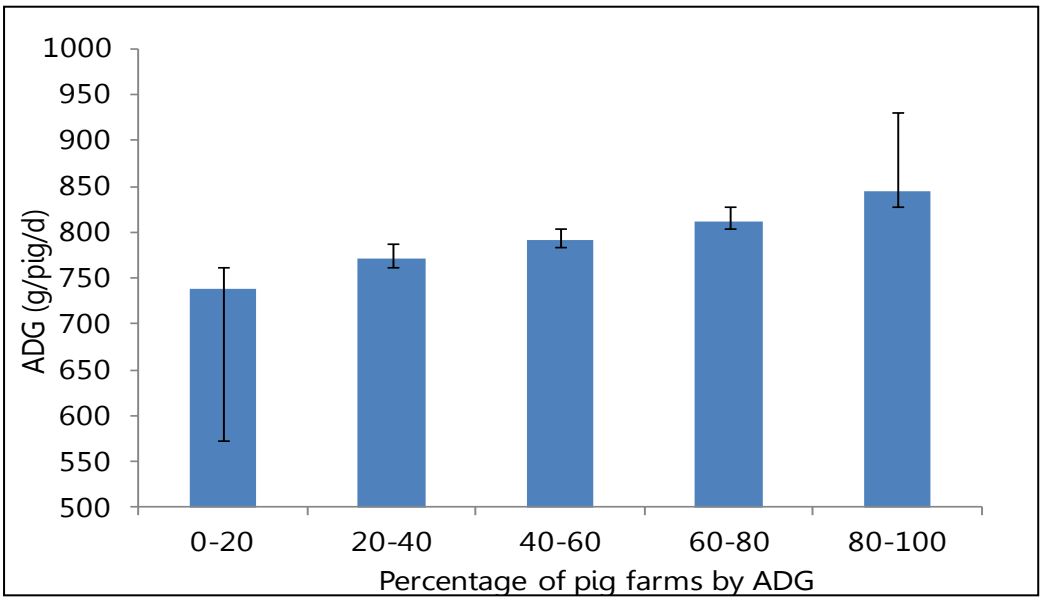


Figure 1.1 Average daily gain (ADG) of pigs between 25 and 118 kg body weight on 887 Dutch growing-finishing pig farms over 2012 expressed per category, ranking all farms in the database in five classes, each representing 20% of the farms. Bars indicate minimum and maximum ADG within each class. Source: Bedrijfsvergelijking AgroVision B.V. (2012).

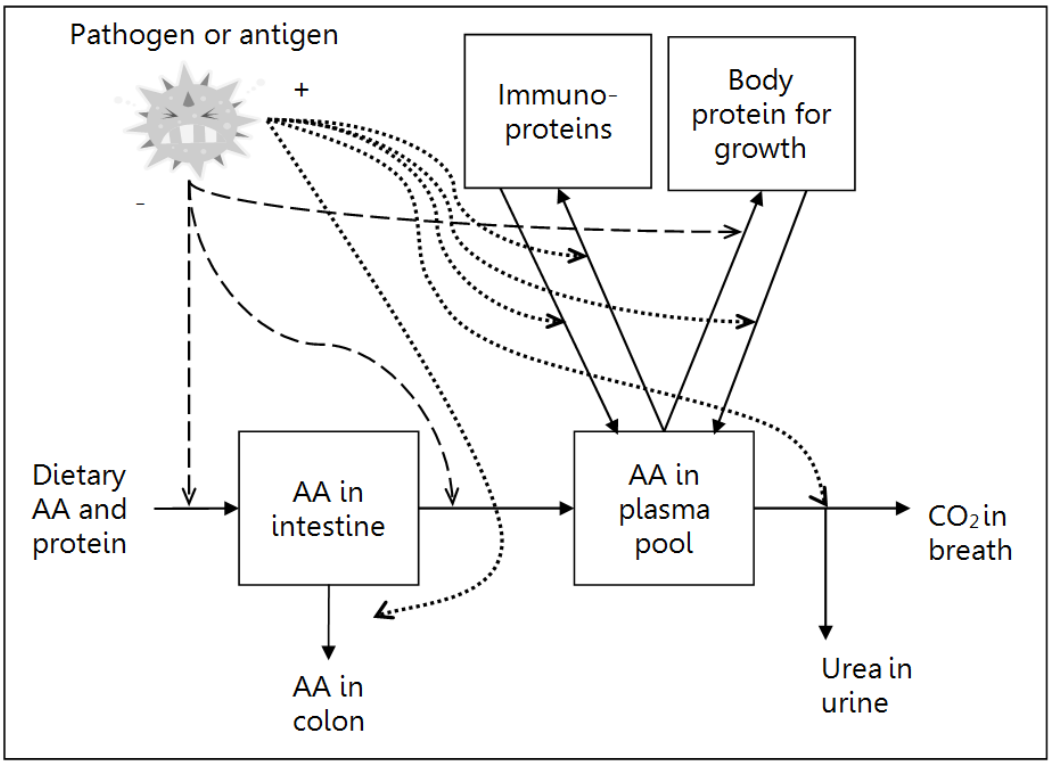


Figure 1.2 Impact of pathogens or antigens on protein and AA metabolism. Black solid arrows indicate nutrient fluxes. Black dotted arrows indicate stimulating effects, dashed arrows indicate inhibiting effects.

Health status

Characterization

In humans, health of an individual is defined as *"a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity*" (WHO, 1948). In animals, the World Organisation for Animal Health (OIE, 2011) defines animal health status as *"the status of a country or a zone with respect to an animal disease, according to the criteria listed in the relevant chapter of the Terrestrial Code dealing with the disease"*. In line with this, in the pig sector, the term SPF (specific pathogen free) is used to categorise farms based on the absence of specific pathogens. Clapperton *et al*. (2008) based their definition of health status of pig farms on *"the presence of major swine diseases that consistently affect both animal welfare and performance"*. Thus in the former three definitions, animal health status merely represents a criterion for the absence or occurrence of disease in a specific animal population. At an individual animal level, a Welfare Quality® assessment protocol for pigs was developed (Dalmau *et al*., 2009), describing criteria for high health as the absence of injuries, diseases, and the absence of pain induced by management procedures. The protocol was developed by 41 universities and research institutes across Europe. In contrast to defining health as the absence of disease, Boersma *et al*. (2009) focussed on the robustness of an animal: *"a healthy animal should be sufficiently robust that it can cope with the causative agents of disease and be healthy again in a short period without remaining disabilities*".

The definitions of health status as mentioned above do not fully cover all aspects of health which are important with respect to growing-finishing pig farms. Therefore, one of the aims of the present thesis was to characterize the health status of growing-finishing pig farms, and not at an individual animal level, with focus on the impact of health status on pig performance, AA metabolism and AA requirements.

In order to define and characterize the health status of growing-finishing pig farms, first, a brainstorm session was held with 13 experts, *i.e.* veterinarians specialized in pigs, scientists in the field of animal nutrition and animal health, and representatives from the commercial pig sector. The outcome of the session is graphically depicted in Figure 1.3. Important factors in characterizing the health status of growing-finishing pig farms included choice of genetic line, factors related to the history of the farm and the pigs during the suckling and post-weaning phase, technical performance, environment (*i.e.* hygienic status or housing conditions), management, and immunological parameters (including pathogen prevalence, blood parameters and other parameters). Further, a selection of pathogens was listed according to two strategies commonly used for establishing or maintaining a high health status (Table 1.1). The first category refers to pathogens which should be eradicated and for which a protection strategy against pathogens should be created to maintain a pathogen free status. The second category refers to pathogens for which a control strategy should be applied to reduce the incidence and damage caused by the disease, *i.e.* the pathogen can be present, but an

outbreak of a disease is prevented or controlled (Heinonen, 2001; Reeves, 2006). Control strategies *e.g.* include hygienic measures, use of vaccines, application of specific feeding strategies, and the use of medicines. Examples of eradication and prevention strategies include depopulation-repopulation, the use of marker vaccines, application of the test and removal principle, *i.e.* removal of animals previously exposed to pathogens based on blood-testing, and restriction of movement of animals within a certain region. It has to be stressed that next to clinical infections, subclinical infections can also induce alterations in metabolism and concomitant losses in production performance (Spurlock, 1997; Sørensen *et al.*, 2006).

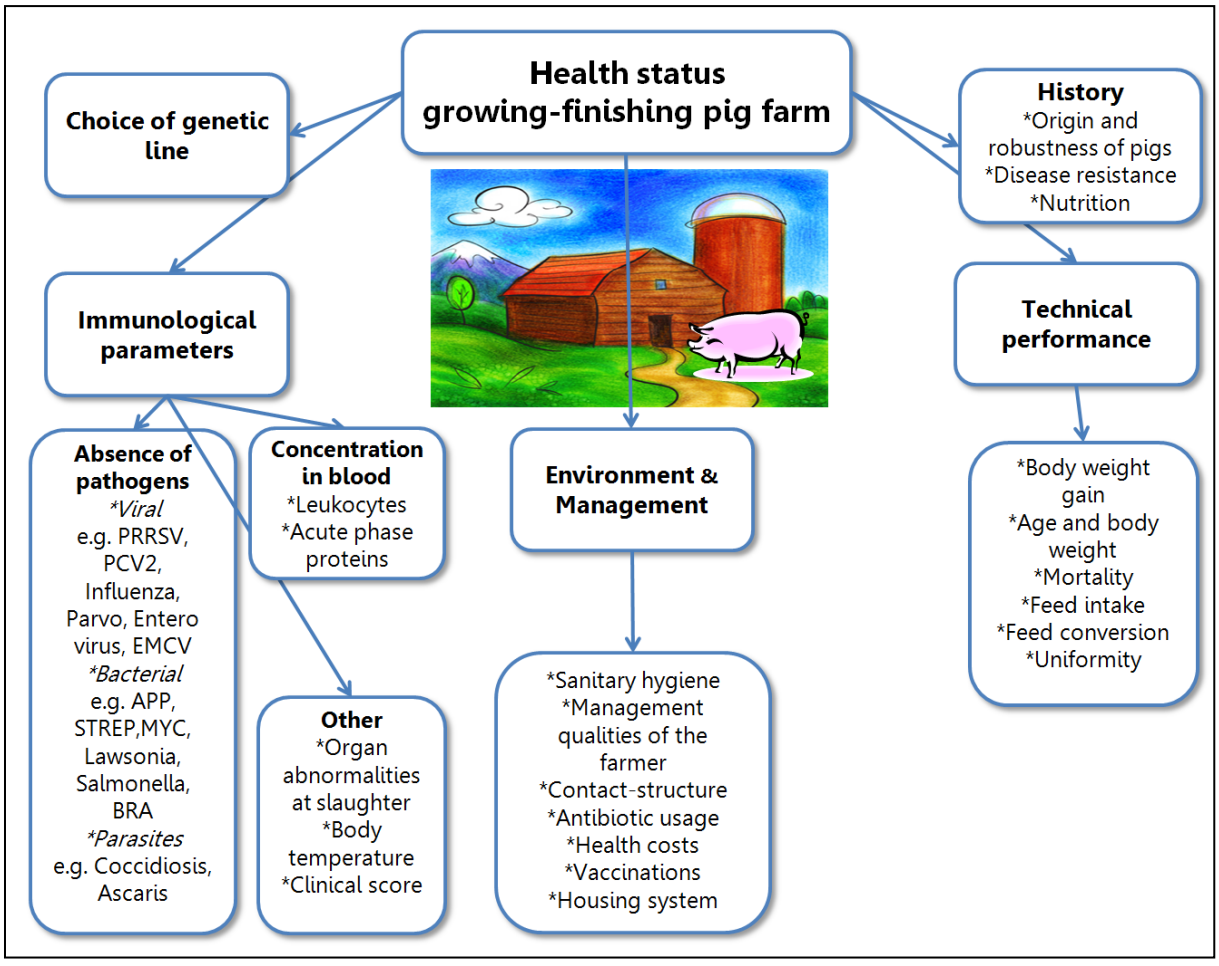


Figure 1.3 Schematic overview of important factors in characterizing the health status of growing-finishing pig farms. Abbreviations used: APP, *Actinobacillus pleuropneumoniae*; BRA, *Brachyspira dysentery*; EMCV, encephalomyocarditis virus; MYC, *Mycoplasma hypneumoniae*; PCV2, Porcine Circovirus type 2; PRRSV, Porcine reproductive and respiratory syndrome virus; STREP, *Streptococcus suis*.

Table 1.1 Pathogens of which its prevalence on farms was listed as a criterion of health status in growing-finishing pig farms, categorized by "Eradication and protection strategies" to create and maintain a pathogen free status, or "Control strategies" to prevent an outbreak and reduce damage.

	Eradication and protection strategy	**Control strategy**
Viral	Swine fever[1]	PRRSV
	Foot-and-mouth disease[1]	PCV2
	Aujeszky's disease[1]	Parvo
	Swine vesicular disease[1]	Enteroviruses
	Transm. gastro-enteritis	EMCV
	Porcine epidemic diarrhoea	Porcine respiratory coronavirus
		Influenza
Bacterial	*Atrophic rhinitis*	*STREP*
	Salmonella cholera suis	*APP*
		MYC
		Haemophilus parasuis
		Lawsonia
		Salmonella
		BRA
		Pasteurella multocida
		Bordetella bronchiseptica
Parasites / protozoa	Scabiës and lice	Coccidiosis
		Ascaris suum

Abbreviations used: APP, *Actinobacillus pleuropneumoniae*; BRA, *Brachyspira dysentery*; EMCV, encephalomyocarditis virus; MYC, *Mycoplasma hypneumoniae*; PCV2, Porcine Circovirus type 2; PRRSV, Porcine reproductive and respiratory syndrome virus; STREP, *Streptococcus suis*.
[1]Listed as notifiable animal disease according to the Dutch Food and Consumer Product Safety Authority.

During the brainstorm session it was concluded that there is a need for characterizing the health status of farms in an objective manner, based on available data, thus without the need for additional on-farm measurements. Next, a questionnaire for veterinarians was developed to gain insight into the prevalence of infectious diseases in Dutch growing-finishing pig farms, and to rank pathogens according to their impact on nutrient requirements of pigs. In this survey, which was conducted in 2010, 12 Dutch veterinarians specialized in growing-finishing pigs were asked to estimate the prevalence of different diseases on pig farms during 2009 and 2010, and to estimate the percentage of pigs that are (sub)clinically infected. Part of the results of this survey is depicted in Figures 1.4 and 1.5. (Sub)clinical infections with ascaris, Porcine circovirus type 2 (PCV2), *Streptococcus suis*, Porcine reproductive and respiratory syndrome virus (PRRSV), *Lawsonia*, *Pasteurella multocida*, and *Bordetella bronchiseptica* are commonly seen in growing-finishing pig farms, and in case of (sub)clinical infections with

Mycoplasma hyopneumoniae, Influenza or PRRSV there is a high prevalence of pigs with clinical symptoms on farms with (sub)clinical infection (Figure 1.4). Based on the brainstorm session and the responses to the questionnaire, it was decided to develop a methodology to objectively characterize health status of growing-finishing pig farms, based on data available and recorded on-farm. Therefore, in the present study, the health status of growing-finishing farms is characterized by the average daily gain (ADG), energy conversion ratio (ECR), mortality, incidence of pleuritis, and incidence of liver and lung abnormalities at slaughter. This concept is presented in Chapter 2.

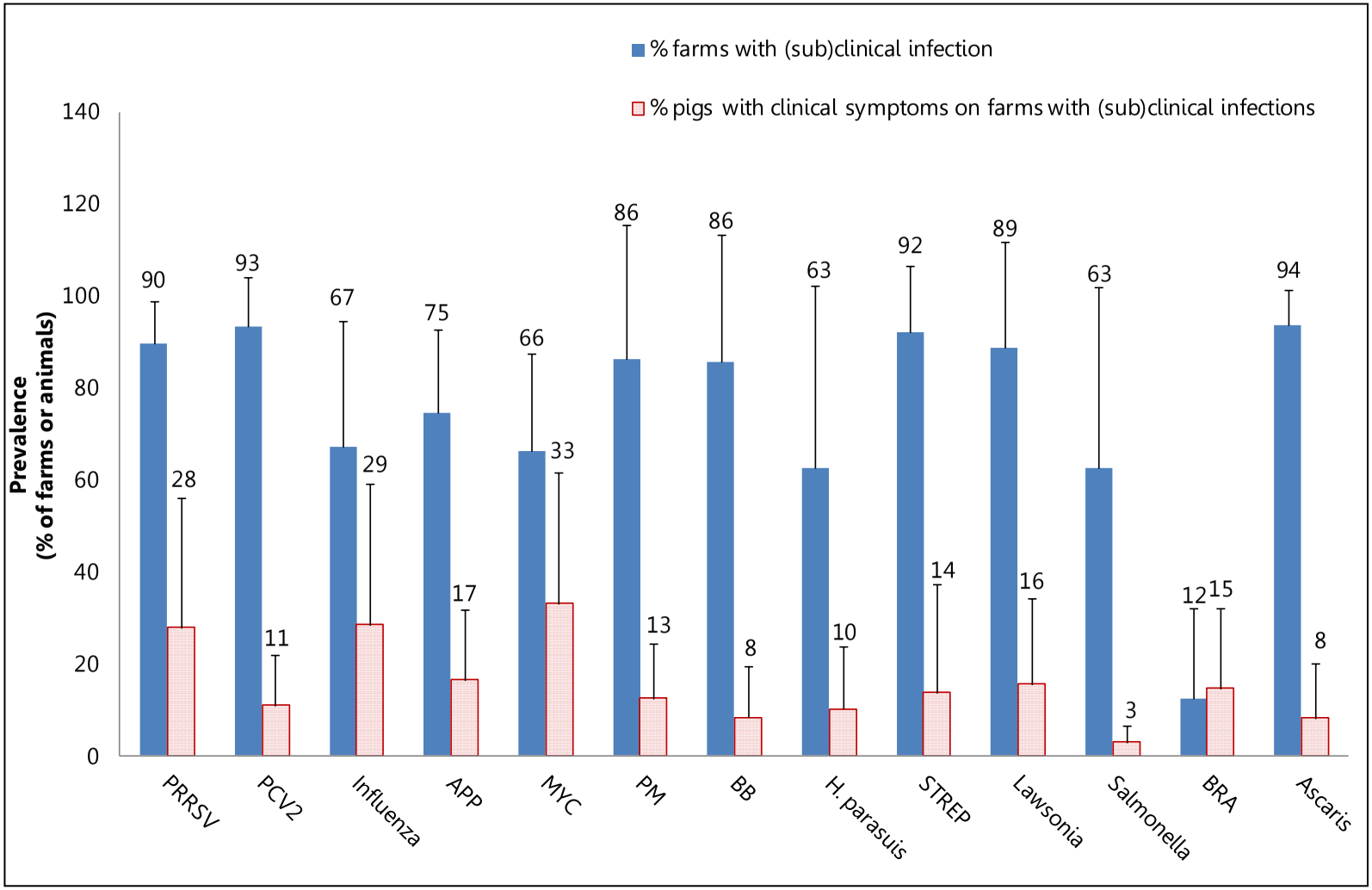


Figure 1.4 Estimated mean prevalence of farms with (sub)clinical infections and prevalence of pigs with clinical symptoms within a population on a farm with (sub)clinical infections in 2009 and 2010, according to expert judgement (n = 12). Error bars indicate SD of the mean estimated prevalence.

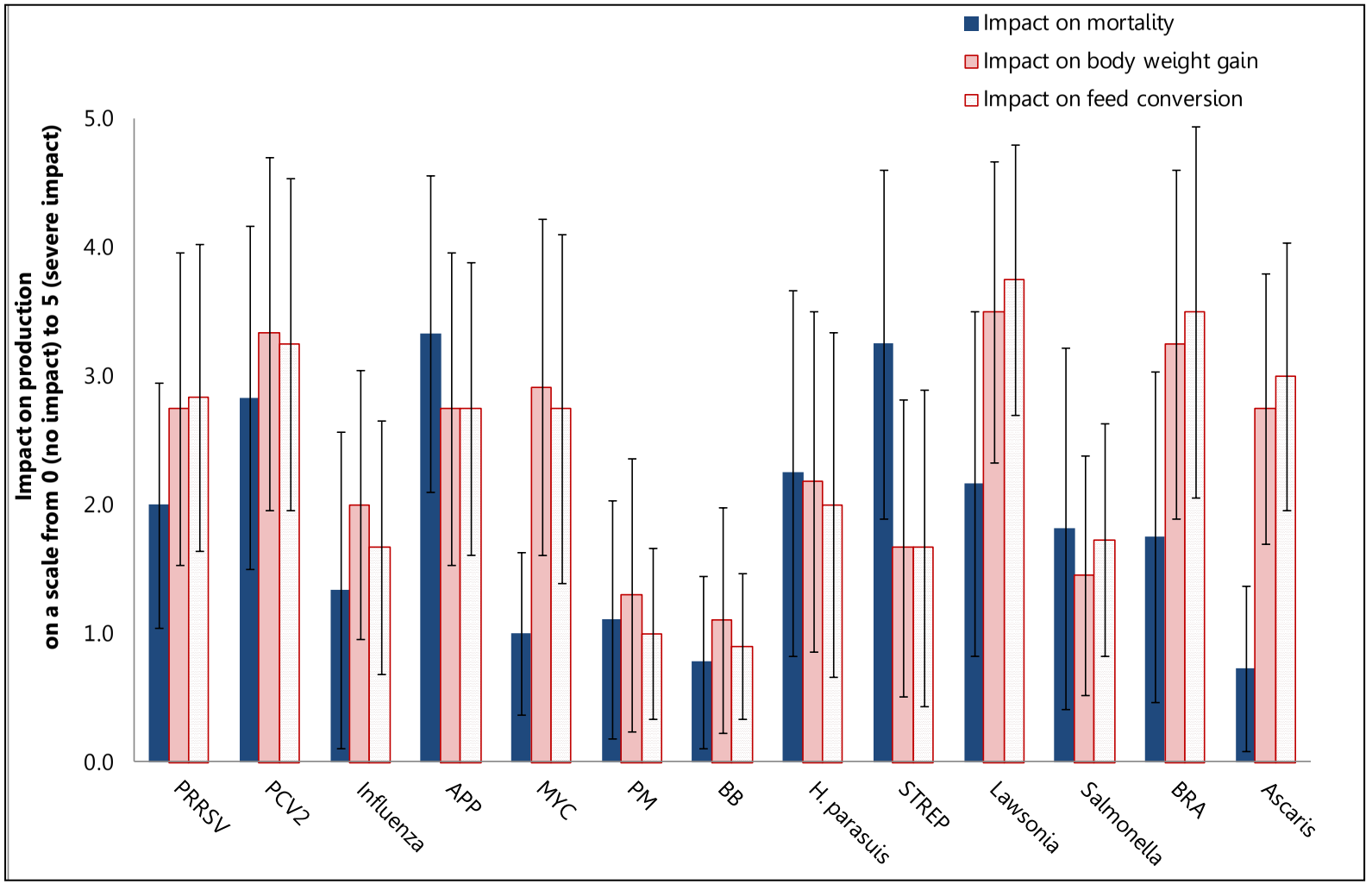


Figure 1.5 Impact of infections on mortality, body weight gain, and feed conversion, of pigs on farms in 2010, according to expert judgement (n = 12). Error bars indicate SD of the mean estimated impact.

Immune system

The immune system acts to protect the host from pathogens, *i.e.* bacteria, viruses, fungi, and parasites, and from other harmful insults. The system recognizes and kills invading pathogens, while sparing the tissue of the host, *i.e.* there must be self-tolerance (Beutler, 2004). The immune system is divided into two functional parts, the innate, or natural immune system, and the acquired immune system, also called specific or adaptive immune system (Figure 1.6). The innate immune system consists of the complement system, epithelial barriers, APP, natural killer cells, and phagocytic cells, *i.e.* granulocytes (neutrophils, basophils, eosinophils), monocytes, macrophages and dendritic cells (Parkin and Cohen, 2001; Calder and Yaqoob, 2012). The humoral part of the innate immune system includes proteins and other molecules that are able to recognize or kill pathogens (Beutler, 2004). The complement system consist of a group of proteins that 1) assists or complements phagocytic cells trough the activation of C3b, which binds to microbes, 2) enhances chemotaxis and the inflammatory response by the production of C5a, and 3) kill Gram-negative bacteria and inactivate viruses by forming the membrane attack complex, consisting of C5 trough C9 (Parkin and Cohen, 2001; Beutler, 2004). The macrophage-derived cytokines TNF-α, IL-1, and IL-6 play an important role in initiating the acute phase response, which is described in more detail in the next paragraph, and in activating the adaptive immune response by stimulating T- and B-lymphocyte proliferation (Calder, 2007a). Acquired or adaptive immunity consists of specific antigen recognition through T- and B-lymphoctyes (Parkin and Cohen, 2001; Calder and Yaqoob, 2012). The acquired immune system takes several days to weeks to develop, whereas the innate immune system provides immediate host defense (Parkin and Cohen, 2001). CD8+ cytotoxic T-lymphocytes can kill infected cells and tumour cells by secretion of cytotoxic enzymes, and CD4+ T-lympocytes, which include Th1, Th2 and regulatory T cells, primarily assist other cells by stimulating phagocytocis, and the maturation of B-cells into plasma cells (Calder and Yaqoob, 2012). B-lymphocytes can carry antigen-specific immunoglobulins and produce immunoglobulins as activated B-cells, *i.e.* plasma cells. Immunoglobulins enhance elements of the innate immune system, including activation of the complement system, phagocytocis, and neutralize toxins (Parkin and Cohen, 2001).

Leukocytes

Leukocytes of healthy pigs in general range between 10 to 18 · 10^9 cells/L, based on reference values developed by the laboratory of the Animal Health Service (GD, The Netherlands). A wider reference value for healthy pigs (Landrace Yorkshire sow · Landrace Duroc boar) between 30-50 kg BW is used, *i.e.* 15.6 to 38.9 · 10^9 cells/L (Klem *et al.*, 2010). Based on GD reference values for pigs, leukocytes include neutrophils (27-62%), lymphocytes (33-56%), monocytes (0-4%), eosinophils (0-7%), and basophils (0-2%). A high leukocyte count cannot be used as a sole indicator of bacterial infections, as the range in reference values in pigs is wide (Klem *et al.*, 2010).

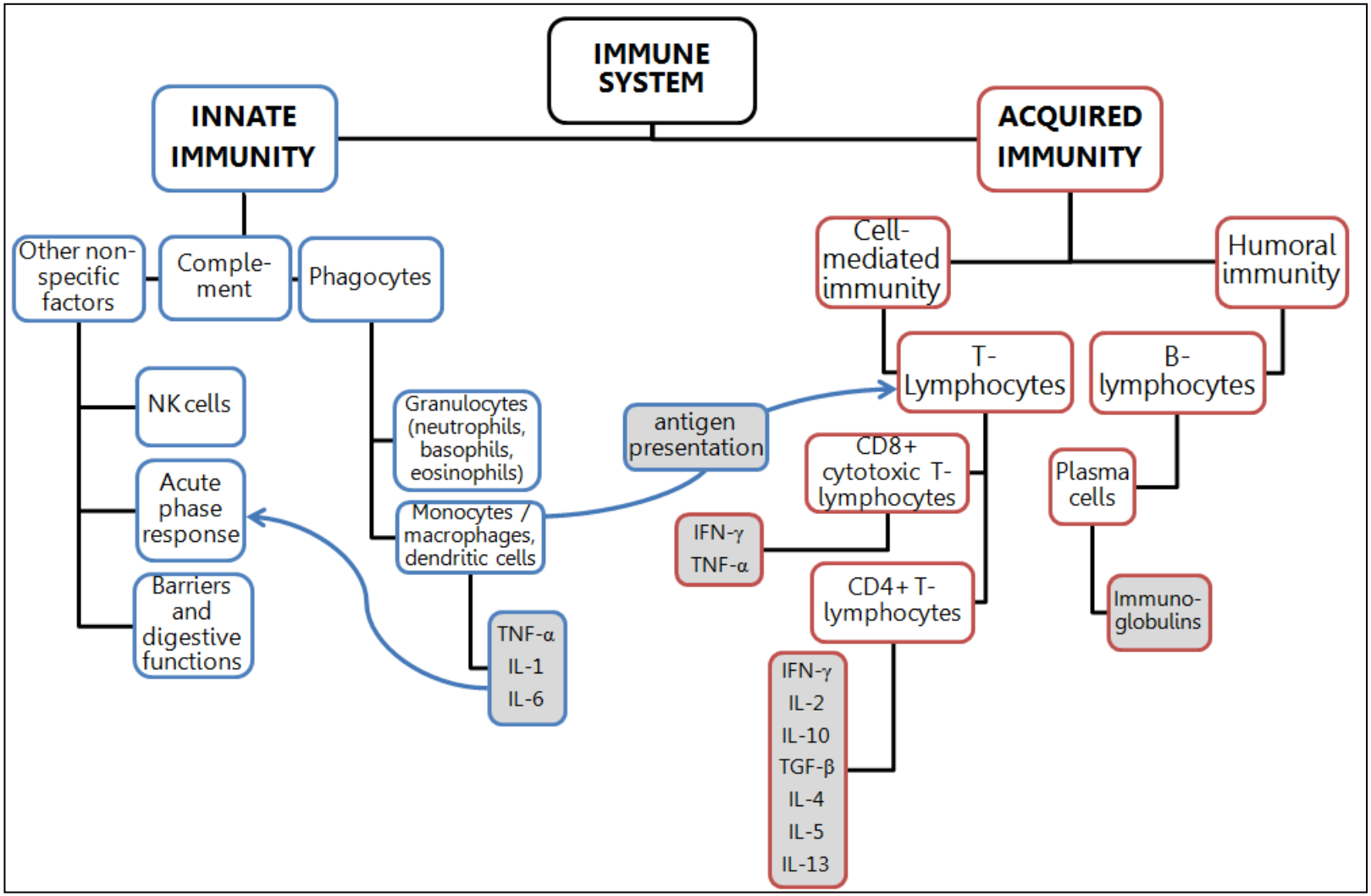


Figure 1.6 Schematic overview of the immune system, adapted from Paajanen (2005). Abbreviations used: APP, acute phase proteins; CD, cluster of differentiation, IFN, interferon; IL, interleukin; NK, natural killer; TGF, transforming growth factor; TNF, tumour necrosis factor.

Besides infection, total leukocyte count and differential counts are affected by breed, age, and housing conditions (Sutherland *et al.*, 2005; Merlot *et al.*, 2012), but not by multiple concurrent stressors, *i.e.* mixing, reduced floor space, and heat (Sutherland *et al.*, 2006). Neutrophils, eosinophils and basophils have granulated cytoplasm and are, therefore, characterized as granulocytes. As part of the cellular component of the innate immune system, neutrophils play an important phagocytic role in destroying microbes (Beutler, 2004) and produce antimicrobial substances (Goldsby *et al.*, 2003). Neutrophils are short-lived, they circulate in peripheral blood for 7-10 h before entering tissues, where they survive for only a few days (Goldsby *et al.*, 2003). An increase in peripheral neutrophils is observed in bacterial infections in pigs (Zhang *et al.*, 1997). Eosinophils (Beutler, 2004) and basophils (Goldsby *et al.*, 2003; Mair *et al.*, 2014) play an important role in parasitic infections, or in allergic reactions. Monocytes circulate in the pheriperal blood for approximately 8 h, after which they migrate into tissues and differentiate into specific tissue macrophages or into dendritic cells (Goldsby *et al.*, 2003). An increase in the percentage of blood monocytes was associated with a decrease in ADG in Large White pigs (Clapperton *et al.*, 2005a). Lymphocytes produce and display antigen binding cell-surface receptors, and are thus part of the cell-mediated adaptive immune response to antigens (Goldsby *et al.*, 2003). Selection and expansion of lymphocytes after antigen exposure, *i.e.* lymphocytes sensitisation, functions to support defence mechanisms against specific antigens (Colditz, 2002). Up to 2 days post *E. coli* challenge in pigs, blood T- and B-lymphocytes decreased compared to pre-challenge, possibly due to increased migration into tissue, *e.g.* spleen and thymus, or apoptosis (Iseri and Klasing, 2013). In addition, a considerable number of lymphocytes are located in the pigs' lung after microbial stimulation (Pabst and Binns, 1994). During a later response to *E. coli* challenge, an increase was observed in CD4+ lymphocytes at day 7, 10 and 14 post-challenge, B-lymphocytes at day 5, 7, and 10, and total leukocyte count at 18 h, 5, 7, and 10 day post-challenge (Iseri and Klasing, 2013).

The acute phase response

The acute phase response, as an important part of the innate immune system, is aimed at restoring homeostasis after infection, inflammation, tissue injury, or stress (Heinrich, 1990; Murata *et al.*, 2004). Monocytes are the primary cells to initiate an acute phase response (Baumann and Gauldie, 1994), but also other cells can be involved, including macrophages, fibroblasts in connective tissues, endothelial cells, and keratinocytes in the outermost layer of the skin (Heinrich, 1990). These cells initiate an acute phase response by the release pro-inflammatory cytokines, mainly interleukin (IL)-6, IL-1, and tumour necrosis factor-α (TNF-α). In turn, a systemic response is induced characterized by APP synthesis in the liver (Heinrich, 1990; Baumann and Gauldie, 1994). In addition, as depicted in Figure 1.7, the systemic response includes the release of adrenocorticotropic hormone (ACTH) from the pituitary gland, leading to an increased secretion of glucocorticoids from the adrenal cortex, which in turn suppress IL-6 levels as a negative feedback mechanism to regulate the acute phase response (Heinrich,

1990). It has been commonplace to discriminate between positive and negative APP, *i.e.* positive APP increase in blood concentration in response to external or internal challenges, such as infection, inflammation, surgical trauma or stress, whereas negative APP decrease in concentration (Murata *et al.*, 2004). An overview of the effect of challenges on APP concentrations in blood is depicted in Table 1.2. In pigs, α1-acid glycoprotein (AGP), C-reactive protein (CRP), haptoglobin, Pig major acute-phase protein (pig-MAP), serum amyloid A (SAA), and fibrinogen have been characterized as positive APP. Albumin, α-lipoprotein or apolipoprotein A1 (ApoA1), and transthyretin have been characterized as negative APP in pigs. Compared to pre-challenge values, post-challenge serum concentrations can reach up to 2400% for haptoglobin, 7520% for CRP, and 600% for pig-MAP, whereas ApoA1 serum concentrations decreased with 80% and albumin with 20% (Table 1.2). SAA is reported to have very short-lived 'all-or-nothing' response to infection, with basal values often below detection limits (Heegaard *et al.*, 2011). In pigs, AGP concentrations did not change after subcutaneous *(s.c.)* turpentine challenge (Lampreave *et al.*, 1994) or after experimentally induced PRRS virus infection (Asai *et al.*, 1999). A decrease in serum ApoA1, and to a lesser extent in serum albumin and transthyretin was observed in a study by Heegaard *et al.* (2011) after *s.c.* turpentine challenge, or experimentally induced infection with *Streptococcus suis* (serotype 2 ribotype I isolate, strain SS02-0119), *Actinobacillus pleuropneumoniae* (serotype 4 isolate) or *Toxoplasma gondii* (isolate SVS P14). Other factors that can increase serum APP are for instance stress caused by transport or housing conditions (Salamano *et al.*, 2008), the occurrence and severity of lesions at slaughter (Pallarés *et al.*, 2008), and tail or ear biting (Salamano *et al.*, 2008; Piñeiro *et al.*, 2013). A reference range of APP were 3.6-183 mg/L for CRP; 0.01-1.31 g/L for haptoglobin, 0.32-2.9 g/L for pig-MAP; and 174-610 mg/L for transthyretin, based on a commercial boar population with a high health status (n = 395 to 397) of approximately 7 months of age (Diack *et al.*, 2011). Recent studies in pigs suggest that APP synthesis is not confined to the liver, as extrahepatic APP gene expression has been demonstrated in pigs experimentally infected with *Actinobacillus pleuropneumoniae* in peripheral lymphoid tissue, spleen, and leukocytes (Skovgaard *et al.*, 2009).

Biological functions of APP

The biological functions of APP have been extensively reviewed (Mackiewicz and Kwang, 1997; Gabay *et al.*, 1999; Suffredini *et al.*, 1999; Murata *et al.*, 2004; Petersen, 2004; Gruys, 2005; Cray, 2012). APP play an important role in a variety of defence-related activities of infection and inflammation (Gabay *et al.*, 1999; Murata *et al.*, 2004). APP function as immunomodulators by acting pro- and/or anti-inflammatory, function as transport proteins, participate in tissue repair, inhibit serine proteinases, *i.e.* enzymes released by pathogens or host tissues, and function as antioxidant by inhibition of hydroxy radical formation, lipid peroxidation, and superoxide production (Mackiewicz and Kwang, 1997; Suffredini *et al.*, 1999; Gruys, 2005). A decrease in plasma

concentration of negative APP possibly functions to divert available AA to the production of positive APP that are required for host defence (Gabay *et al.*, 1999).

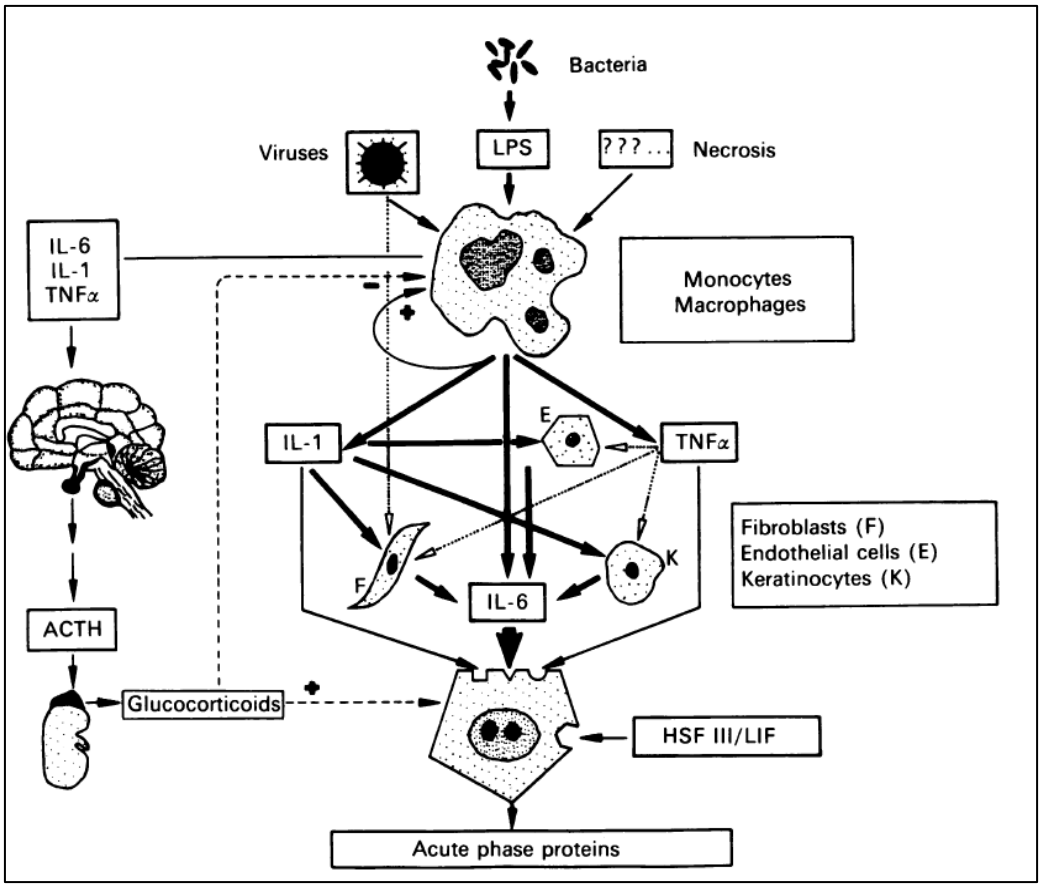


Figure 1.7 Regulation of hepatic acute phase protein synthesis by inflammatory mediators (Heinrich, 1990). Abbreviations used: ACTH, adrenocorticotropic hormone ; HSF III, hepatocyte stimulating factor; IL, interleukin; LIF, leukaemia inhibitory factor; LPS, lipopolysaccharide; TNF, tumour necrosis factor.

Positive APP

Alpha 1-acid glycoprotein

AGP functions locally by reducing tissue damage especially in epithelial and endothelial cells, and systemically by binding to drugs (Murata *et al.*, 2004) and lipopolysaccharide (LPS) (Cray, 2012). In addition, AGP, as immunomodulator, has anti-inflammatory properties, including the inhibition of neutrophil activity, lymphocyte proliferation, and complement system activity (Fournier *et al.*, 2000). C-reactive protein

A major function of CRP is to bind to phosphocholine, by which pathogens and phospholipid constituents of damaged cells are recognized and their elimination is initiated (Kushner and Rzewnicki, 1994; Mackiewicz and Kwang, 1997; Gabay *et al.*, 1999). CRP activates the complement system, and enhances phagocytosis (Gabay *et al.*, 1999; Gruys, 2005). Other pro-inflammatory effects of CRP include the induction of

inflammatory cytokines and tissue factor, which enables cells to initiate blood coagulation (Gabay *et al.*, 1999). CRP, however, also has anti-inflammatory properties including prevention of adhesion of neutrophils to endothelial cells, inhibition of the production of superoxide by neutrophils, and induce the production of anti-inflammatory cytokines like IL-1 receptor antagonist by mononuclear cells (Gabay *et al.*, 1999).

Haptoglobin

A major function of haptoglobin is the binding to and clearance of haemoglobin (Mackiewicz and Kwang, 1997; Gruys, 2005; Cray, 2012), by which the formation of reactive oxygen by iron and iron compounds (Gutteridge, 1987), and bacterial growth (Hossein Sadrzadeh and Bozorgmehr, 2004) is inhibited. Other immunomodulatory effects of haptoglobin include the inhibition of granulocyte chemotaxis, phagocytosis, mast cell proliferation, maturation of epidermal Langerhans cells, *i.e.* antigen presenting cells of the skin, and suppress T-cell proliferation as reviewed by Murata *et al.* (2004). In addition, haptoglobin plays an important role in tissue repair by stimulating angiogenesis, *i.e.* the formation of blood vessels (Cid *et al.*, 1993).

Fibrinogen

Fibrinogen plays a major role in haemostasis, *i.e.* the process that stops bleeding, by aiding in clot-formation (Mackiewicz and Kwang, 1997; Gruys, 2005), and provides a matrix for the migration of inflammatory cells which enhance tissue repair (Murata *et al.*, 2004).

Pig major acute-phase protein

Pig-MAP, or inter-a-trypsin inhibitor heavy chain 4, has been reported to be a counterpart of a human serum protein denominated PK-120, and is a major serum APP in pigs (González-Ramón *et al.*, 1995; González-Ramón *et al.*, 2000). The homology to PK-120 implies that pig-MAP is a substrate for plasma kallikrein (Nishimura *et al.*, 1995), a serine protease that plays a key role in repair of damaged tissue, by cleaving kinogen into bradykinin (Goldsby *et al.*, 2003). Kinins are inflammatory peptides that, for instance, increase vascular permeability, cause vasodilation, induce contraction of smooth muscle, and act directly on the complement system (Goldsby *et al.*, 2003). Pig-MAP shows homology with the ITI superfamily of serum trypsin inhibitors, however, pig-MAP does not inhibit trypsin (González-Ramón *et al.*, 1995).

Serum amyloid A

SAA consists of a family of apolipoproteins that influences high-density lipoprotein-cholesterol transport, *e.g.* by binding and neutralizing LPS (Gruys, 2005). It has pro-

inflammatory and anti-inflammatory properties (Suffredini *et al.*, 1999). SAA attracts monocytes, lymphocytes, and granulocytes by inducing chemotaxis (Suffredini *et al.*, 1999; Gruys, 2005), and stimulates the adhesion to blood vessels. In addition, SAA activates leukocytes to kill microbes, and has antifungal activity (Suffredini *et al.*, 1999). SAA acts as a mediator by activation monocytes and macrophages to produce cytokines and induce inflammation (Song *et al.*, 2009; Lee *et al.*, 2013). At high serum concentrations, SAA has anti-inflammatory properties, including the inhibition of lymphocyte and endothelial cell proliferation, and of platelet aggregation (Murata *et al.*, 2004). In addition, SAA inhibits the respiratory burst of leukocytes that produce free radicals (Gruys, 2005).

Negative APP

Albumin

Albumin is the most abundant protein in serum and two of its major functions are to act as a regulator of osmotic pressure and as binder or transporter of substrates (Rothschild *et al.*, 1969), including fatty acids, minerals, AA and proteins, including bacterial proteins, bilirubin pigment, vitamins, hormones, and drugs (Fanali *et al.*, 2012). In addition, albumin exerts anti-oxidant activity, for instance by providing substrates that reduce free radicals, and functions as a depot for endogenous and exogenous compounds (Fanali *et al.*, 2012). A decrease in albumin induces a temporary increased availability of free hormones that bind to albumin (Gruys, 2005).

Apolipoprotein A-I

ApoA1 is the major protein component of α-lipoprotein or high density lipoprotein (Carpintero *et al.*, 2005). Under normal conditions, ApoA1 inhibits cytokine production in monocytes by binding to the activating factor on stimulated T lymphocytes. During acute inflammation, however, ApoA1 concentration decreases to allow the production of pro-inflammatory cytokines by monocytes (Burger and Dayer, 2002).

Transthyretin

Transthyretin, also known as thyroxin-binding prealbumin, serves as a transport protein by binding to thyroxin, *i.e.* the most abundant thyroid hormone in blood, and forms a complex with retinol-binding protein to aid the transport of vitamin A (Raz and Goodman, 1969; Schreiber and Richardson, 1997). As with albumin, a decrease in transthyretin induces a temporary increased availability of free hormones that bind to this protein (Gruys, 2005). Transthyretin inhibits IL-1 production by monocytes and endothelial cells; a decrease in concentration is thus suggested to be pro-inflammatory (Gabay *et al.*, 1999).

Table 1.2 Acute phase protein concentrations in serum of healthy and immune challenged growing pigs.

Immune parameter	Effect	Pre-challenge Mean ± SD, or range	Post-challenge peak, Mean ± SD, or range	Immune challenge	Reference
Positive APP					
AGP, µg/mL	Not affected	Range 500 to 1000	Range 500 to 1000	PRRS virus challenged (n = 7) *vs.* control (n = 3)	Asai *et al.* (1999)
CRP, µg/mL	At slaughter	85 ± 11.7	253 ± 218.0 (200%)	Characterized by poor growth (n = 20) *vs.* clinically healthy (n = 352)	Chen *et al.* (2003)
	Max at d 1-2 PC	Range 10 (DL) to 20	Range 50 to 110	*s.c.* injection with 0.3 ml turpentine oil/kg BW, *vs.* basal (n = 6)	Carpintero *et al.* (2005)
	Max at d 6 PC	18 ± 11.4[1]	35 ± 15 (90%)[1]	*A. pleuropneumoniae* challenged, serotype 4 isolate *vs.* basal (n = 12). Similar response for *T. gondii* challenged, isolate SVS P14 *vs.* basal (n = 5)	Heegaard *et al.* (2011)
	Max at d 2 PC	18 ± 11.4[1]	55 ± 23 (210%)[1]	*S. suis*, serotype 2 ribotype I isolate, strain SS02-0119 *vs.* basal (n = 5)	Heegaard *et al.* (2011)
	Max at d 3 PC	18 ± 11.4[1]	70 ± 8 (290%)[1]	*s.c.* injection with 0.3 ml turpentine oil/kg BW, *vs.* basal (n = 5)	Heegaard *et al.* (2011)
		5, 0 and 25[2]	139, 111 and 229 (2680%)[2]	Pigs positive to specific IgM and IgG of porcine circovirus type 2 of one farm (n = 10) *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
		5, 0 and 25[2]	381, 233 and 415 (7520%)	Pigs with *M. hyopneumoniae* infection (n = 10) *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)

		5, 0 and 25[2]	203, 175 and 207 (3960%)	Pigs with signs of inflammation due to tail and ear bites, arthritis, rectal prolaps, or ulcerated umbilical hernia (n = 16 *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
	At d1 PC	1.1 ± 1.6	15 ± 5.4 (1260%)	*A. pleuropneumoniae* challenged, serotype 5 isolate L20, *vs.* basal (n = 11)	Skovgaard *et al.* (2009)
Haptoglobin, mg/mL	Max at d 7 PC	2.5 ± 2.5[1]	7.9 ± 2.0[1] (220%)	PRRS virus challenged (n = 7) *vs.* control (n = 3)	Asai *et al.* (1999)
		Range 0.4 to 0.8	Range 1.4 to 3.6	*s.c.* injection with 0.3 ml turpentine oil/kg BW, *vs.* basal (n = 6)	Carpintero *et al.* (2005)
	At slaughter	1.4 ± 0.1	2.2 ± 2.6 (60%)	Characterized by poor growth (n = 20) *vs.* clinically healthy (n = 352)	Chen *et al.* (2003)
	Max at d 3 PC	0.7 ± 0.7[1]	1.4 ± 1.0 (100%)[1]	*A. pleuropneumoniae* challenged, serotype 4 isolate *vs.* basal (n = 12)	Heegaard *et al.* (2011)
	Max at d 8 PC	0.7 ± 0.7[1]	4.0 ± 2.0 (470%)[1]	*T. gondii* challenged, isolate SVS P14 *vs.* basal (n = 5)	Heegaard *et al.* (2011)
	Max at d 2 PC	0.7 ± 0.7[1]	3.9 ± 0.6 (460%)[1]	*S. suis*, serotype 2 ribotype I isolate, strain SS02-0119 *vs.* basal (n = 5)	Heegaard *et al.* (2011)
	Max at d 3 PC	0.7 ± 0.7[1]	2.8 ± 0.7 (300%)[1]	*s.c.* injection with 0.3 ml turpentine oil/kg BW, *vs.* basal (n = 5)	Heegaard *et al.* (2011)
	Max at d 4 PC	0.4 ± 0.6[1]	4.1± 1.1 (930%)[1]	*A. pleuropneumoniae* challenged, serotype 2 (strain 700/89) *vs.* basal (n = 10)	Hultén *et al.* (2003)
	Max at d 4 PC	0.2 ± 0.2	3.6 ± 0.9 (1400%)[1]	*S. suis* serotype 2 (strain 93) *vs.* basal (n = 8)	Knura-Deszczka *et al.* (2002)
	At d 13 PW, but not d 34	0.4	1.0 (150%)	Lower sanitary status and absence of antibiotic-supplementation (n = 20) *vs.* control (n = 20)	Le Floc'h *et al.* (2006)

	At d 13 and 43 PW, but not at 36 d	0.8 at d 13, 0.6 at d 43	1.4 (80% at d 13 and 130% at d 43)	Moderate inflammation due to lower sanitary status and absence of antibiotic-supplementation (n = 80) *vs.* control (n = 80)	Le Floc'h *et al.* (2010)
	Max at d 2 PC	0.8 ± 0.4[1]	2.2 ± 0.4[1] (180%)	*i.v.* CFA challenge (n = 10) *vs.* control (n = 10)	Le Floc'h *et al.* (2008)
	At d 7 PC	0.4 ± 0.3	0.9 ± 0.3 (130%)	*i.m. E. coli* LPS challenge with increasing doses *vs.* basal (n = 15-17)	Litvak *et al.* (2013a)
	Not affected	Range 0.4 to 5	Range 5 to 11	*A. pleuropneumoniae* challenged, serotype 5 isolate L20, *vs.* basal (n = 11)	Skovgaard *et al.* (2009)
		0.2, 0.03 and 0.5[2]	5, 4 and 6 (2400%)[2]	Pigs positive to specific IgM and IgG of porcine circovirus type 2 of one farm (n = 10) *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
		0.2, 0.03 and 0.5[2]	3.5, 3 and 5 (1650%)[2]	Pigs with *M. hyopneumoniae* infection (n = 10) *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
		0.2, 0.03 and 0.5[2]	4, 2.7 and 5.8 (1900%)[2]	Pigs with signs of inflammation due to tail and ear bites, arthritis, rectal prolaps, or ulcerated umbilical hernia (n = 16 *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
pig-MAP, mg/mL	Max at d 2-3 PC	0.6	Range 3 to 3.5 (480%)	*s.c.* injection with 0.3 ml turpentine oil/kg BW, *vs.* basal (n = 6)	Carpintero *et al.* (2005)
	Max at d 2 PC	Range 0.5 to 1.0	Range 4.5 to 7.0 (600%)	*A. pleuropneumoniae* challenged, serotype 5b, biotype 1 *vs.* basal (n = 3)	Carpintero *et al.* (2005)
	Max at d 4-6 PC	0.4, range 0.4 to 0.7	Range 2.0 to 13.0 (3150%)	*Iv*injection of *S. suis* serotype 2 strain 93 *vs.* basal (n = 8)	Carpintero *et al.* (2005)
	Max at d 3 PC	0.9 ± 0.4[1]	3.0 ± 1.3 (230%)[1]	*A. pleuropneumoniae* challenged, serotype 4 isolate (n = 12)	Heegaard *et al.* (2011)

	Max at d 8 PC	0.9 ± 0.4[1]	2.5 ± 0.3 (180%)[1]	*T. gondii* challenged, isolate SVS P14 *vs.* basal (n = 5)	Heegaard *et al.* (2011)
	Max at d 5 PC	0.9 ± 0.4[1]	4.5 ± 1.5 (400%)[1]	*S. suis*, serotype 2 ribotype I isolate, strain SS02-0119 *vs.* basal (n = 5)	Heegaard *et al.* (2011)
	Max at d 3 PC	0.9 ± 0.4[1]	2.5 ± 1.0 (180%)[1]	*s.c.* injection with 0.3 ml turpentine oil/kg BW, *vs.* basal (n = 5)	Heegaard *et al.* (2011)
		0.8, 0.6 and 1.2[2]	3.3, 2.5 and 3.7 (310%)[2]	Pigs positive to specific IgM and IgG of porcine circovirus type 2 of one farm (n=10) *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
		0.8, 0.6 and 1.2[2]	2.2, 1.3 and 3.3 (180%)[2]	Pigs with *M. hyopneumoniae* infection (n = 10) *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
		0.8, 0.6 and 1.2[2]	2.6, 1.7 and 3.6 (230%)[2]	Pigs with signs of inflammation due to tail and ear bites, arthritis, rectal prolaps, or ulcerated umbilical hernia (n = 16 *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
SAA, µg/mL	Max at d 3 PC	< 6 DL[3]	120 ± 75 (ND)[1]	*A. pleuropneumoniae* challenged, serotype 4 isolate *vs.* basal (n = 12)	Heegaard *et al.* (2011)
	Max at d 8 PC	< 6 DL	90 ± 20 (ND)[1]	*T. gondii* challenged, isolate SVS P14 *vs.* basal (n = 5)	Heegaard *et al.* (2011)
	Max at d 3 PC	< 6 DL	240 ± 130 (ND)[1]	*S. suis*, serotype 2 ribotype I isolate, strain SS02-0119 *vs.* basal (n = 5)	Heegaard *et al.* (2011)
	Max at d 3 PC	< 6 DL	340 ± 100 (ND)[1]	*s.c.* injection with 0.3 ml turpentine oil/kg BW, *vs.* basal (n = 5).	Heegaard *et al.* (2011)
	Max at d 2 PC	< 19 DL	520 ± 411 (ND)[1]	*A. pleuropneumoniae* challenged, serotype 2 (strain 700/89) *vs.* basal (n = 10)	Hultén *et al.* (2003)
	At d1 PC	<2.5 DL	224 ± 49 (ND)	*A. pleuropneumoniae* challenged, serotype 5 isolate L20, *vs.* basal (n = 11)	Skovgaard *et al.* (2009)

Fibrinogen	At d 7 PC	1.6 ± 0.4	2.5 ± 0.4 (60%)	*i.m. E. coli* LPS challenge with increasing doses *vs.* basal (n = 15-17)	Litvak *et al.* (2013a)
Negative APP					
Albumin, mg/mL	Not affected	Range 15-32	Range 15-32	*A. pleuropneumoniae, M hyos, S. suis, T. gondii,* or *s.c.* turpentine challenge *vs.* basal	Heegaard *et al.* (2011)
	At d 7 PC	34.0 ± 2.0	29.8 ± 2.0 (-10%)	*i.m. E. coli* LPS challenge with increasing doses *vs.* basal (n = 15-17)	Litvak *et al.* (2013a)
		32, 28 and 34[2]	25, 22 and 30 (-20%)[2]	Pigs positive to specific IgM and IgG of porcine circovirus type 2 of one farm (n = 10) *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
	Not affected	32, 28 and 34[2]	32, 31 and 38[2]	Pigs with *M. hyopneumoniae* infection (n = 10) *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
	Not affected	32, 28 and 34[2]	28, 26 and 31[2]	Pigs with signs of inflammation due to tail and ear bites, arthritis, rectal prolaps, or ulcerated umbilical hernia (n = 16 *vs.* pigs from SPF farm (n = 17)	Parra *et al.* (2006)
ApoA1, mg/mL	Min at d 2-3 PC	2.7, range 2.1 to 3.2	1.3 (-50%), range 1.2 to 1.5	*s.c.* injection with 0.3 ml turpentine oil/kg BW, *vs.* basal (n = 6).	Carpintero *et al.* (2005)
	Min at d 2- 3 PC	2.3, range 1.8 to 3.1	0.8 (-70%), range 0.5 to 1.0	*A. pleuropneumoniae* challenged, serotype 5b, biotype 1 *vs.* basal (n = 3)	Carpintero *et al.* (2005)
	Min at d 2- 3 PC	3.1, range 2.4 to 3.9	0.6 (-80%), range 0.5 to 1.23	*Iv* injection of *S. suis* serotype 2 strain 93 *vs.* basal (n = 8)	Carpintero *et al.* (2005)
	Min at d 3 PC	1.9 ± 0.7[1]	1.0 ± 0.3 (-50%)[1]	*A. pleuropneumoniae* challenged, serotype 4 isolate (n = 12). Similar response for *S. suis*, serotype 2 ribotype I isolate, strain SS02-0119 *vs.* basal (n = 5)	Heegaard *et al.* (2011)
	Min at d 8 PC	1.9 ± 0.7[1]	1.6 ± 0.2(-20%)[1]	*T. gondii* challenged, isolate SVS P14 *vs.* basal (n = 5).	Heegaard *et al.* (2011)

	Min at d 3 PC	1.9 ± 0.7[1]	1.6 ± 0.6 (-20%)[1]	*s.c.* injection with 0.3 mL turpentine oil/kg BW, *vs.* basal (n = 5).	Heegaard *et al.* (2011)
Transthyretin, µg/mL	Not affected	Range 10 to 45	Range 10 to 45	*A. pleuropneumoniae, M. hyos, S. suis, T. gondii,* or *s.c.* turpentine challenge *vs.* basal	Heegaard *et al.* (2011)
	Not affected	Range 130 to 180[1]	Range 20 to 320[1]	*A. pleuropneumoniae* challenged, serotype 5 isolate L20, *vs.* basal	Skovgaard *et al.* (2009)

AGP, alpha (α)-1 acid glycoprotein; ApoA1, apolipoprotein A-I; CRP, C-reactive protein; M. hyos, *Mycoplasma hyosynoviae*; PC, post-challenge; DL, detection limit; *i.m.*, intramuscular; ND, not determined; Pig-MAP, Pig major acute-phase protein; PW, post-weaning; SAA, Serum amyloid A; *s.c.*, subcutaneous.
[1]Mean and SD values derived from graphical presentation in the original paper.
[2]Median, 25th and 75th percentage.
[3]SAA basal values often below detection limit (6 µg/mL).

Role of amino acids in the immune system

The role of AA in the immune system is extensively reviewed by Li *et al.* (2007). They have several functions in the immune system, *e.g.* stimulation of lymphocyte proliferation, regulation or activation of cytokine production, inhibition of apoptosis, aid in immune defence by being antiviral and kill pathogens, some function as fuel for cells of the immune system, and are involved as antioxidant or in regulating cellular redox state.

As the only precursor for nitric oxide, Arg plays an role in host defence (Beisel, 1996) and functions as a regulator in cardiovascular functions (Lorin *et al.*, 2014). The branched-chained AA (BCAA) Leu, Ile, and Val are important for lymphocyte proliferation, stimulate the production of proteins including cytokines, immunoglobulins and antibodies (Calder, 2006). Cys is, together with Gln and Gly, a precursor of glutathione, which has several functions including detoxification of substances, antioxidant *e.g.* by neutralizing reactive oxygen species which are increasingly produced during inflammation, and modulation of cell proliferation (DeLeve and Kaplowitz, 1991; Lu, 2009). Cys can be produced from Met via homocysteine, and sulphur AA are suggested to play an important role in immune system functioning (Grimble, 2006; Kim *et al.*, 2012; Litvak *et al.*, 2013a,b; Rakhshandeh *et al.*, 2014). APP are particularly high in Phe, Trp, and Tyr, and these AA are thus suggested to be required for APP synthesis (Reeds *et al.*, 1994). In addition, Trp plays an important role in cell division (Mellor and Munn, 2004) and as immune modulator through several Trp metabolites, such as the extrahepatic enzyme indoleamine 2,3 dioxygenase (IDO) (Chen and Guillemin, 2009; de la Fuente *et al.*, 2012). Gln is an important regulator of immune function by regulating monocyte function, *e.g.* increase IL secretion, stimulate phagocytosis and antigen presentation, by regulating lymphocyte function, *e.g.* stimulating INF-γ secretion and inhibiting apoptosis, and activating and stimulating natural killer cells (Roth *et al.*, 2002; Roth, 2007). In addition, Gln plays an important role by interfering with Arg and NO metabolism, and by being a precursor of glutathione (Roth, 2007). Furthermore, Gln is a major energy source for rapidly dividing cells, moreover lymphocyte proliferation is dependent on extracellular Gln concentrations (Newsholme, 2001). It also has an important function in the gastrointestinal tract, as do Arg, Glu, sulphur AA, Gly, and Lys (Wang *et al.*, 2009). Gln for instance prevents villi damage in post-weaning piglets (Wu *et al.*, 1996). Thr, Ser, Pro, and Cys are required for the synthesis of mucins, and thereby promote the function of the the gastrointestinal tract (Faure *et al.*, 2006). In addition, Thr plays an important role in the production of humoral antibodies and immunoglobulins in pigs (Defa *et al.*, 1999; Wang *et al.*, 2006b). In the paragraph "Nutritional modulation of the immune system" more details on the function of AA in the immune system are provided, with emphasis on the effect of dietary AA supply.

Nutritional costs of immune system activation

Protein metabolism associated costs

During immune system activation in animals, nutrients are redistributed from anabolic and maintenance processes towards processes involved in immunity and disease resistance (Klasing and Johnstone, 1991; Spurlock, 1997). During this process, a cascade of cytokine induced metabolic alterations occur, including anorexia, increased breakdown and decreased synthesis of skeletal muscle protein (Zamir *et al.*, 1992; Breuille, 1999), increased hepatic acute APP synthesis, and increased deamination of glucogenic AA (Klasing and Johnstone, 1991; Lochmiller and Deerenberg, 2000; Le Floc'h *et al.*, 2004). In humans, the metabolic response due to immune system activation results in a net loss of body protein up to 20%, primarily from skeletal muscle, due to an increase in protein catabolism and a reduction in anabolic response to feeding (Biolo *et al.*, 1997). In pigs, immune system activation induced by repeated LPS challenge reduced body protein deposition with 3% to 20% (de Ridder *et al.*, 2012; Litvak *et al.*, 2013a). Immune system activation induced by a rearing scheme that maximized the pigs' exposure to pathogens reduced the *Longissimus* muscle area with 3% to 20%, compared to pigs on a rearing scheme that minimized pathogen exposure (Williams *et al.*, 1997c). In line, a reduction in muscle weight of more than 20% was observed in PRRSV challenged pigs compared to control (Escobar *et al.*, 2004). Protein synthesis in the liver, however, is increased (Wolfe, 1999). In addition, changes in protein metabolism during immune system activation are associated with fever (Kluger, 1991; Netea *et al.*, 2000), pain, and a change in physical activity (Johnson, 2002), a reduction in feed intake (Williams *et al.*, 1997a; Johnson, 1998; Sandberg *et al.*, 2006; Daiwen *et al.*, 2008; Pastorelli *et al.*, 2012), and reduction in growth performance (Spurlock *et al.*, 1997; Williams *et al.*, 1997a; Daiwen *et al.*, 2008) (Figure 1.8). The simultaneous decrease in feed intake and thus in the quantity of AA ingested and the reduction in muscle protein deposition leads to a decreased AA utilization for body protein deposition. In contrast, however, the utilization of specific AA for the synthesis of proteins for the immune system increases. Consequently, the optimal AA profile required for growth and immune function may change during an acute or chronic state of immune system activation, *e.g.* by continuous exposure to pathogens.

Amino acid costs

Recent studies in pigs revealed that immune system activation by intramuscular *(i.m.)* LPS administration increases the optimal dietary Met to Met + Cys ratio (Litvak *et al.*, 2013a) and dietary sulphur AA to Lys ratio (Kim *et al.*, 2012), and reduces the efficiency of Trp utilization for body protein deposition (de Ridder *et al.*, 2012). These findings indicate that the requirements for AA in growing pigs may be affected by health status. It is likely that the requirement of certain AA, in particular aromatic AA, increase for the production of APP during immune system activation. For the synthesis of APP, AA are provided either from dietary protein or from breakdown of skeletal muscle protein.

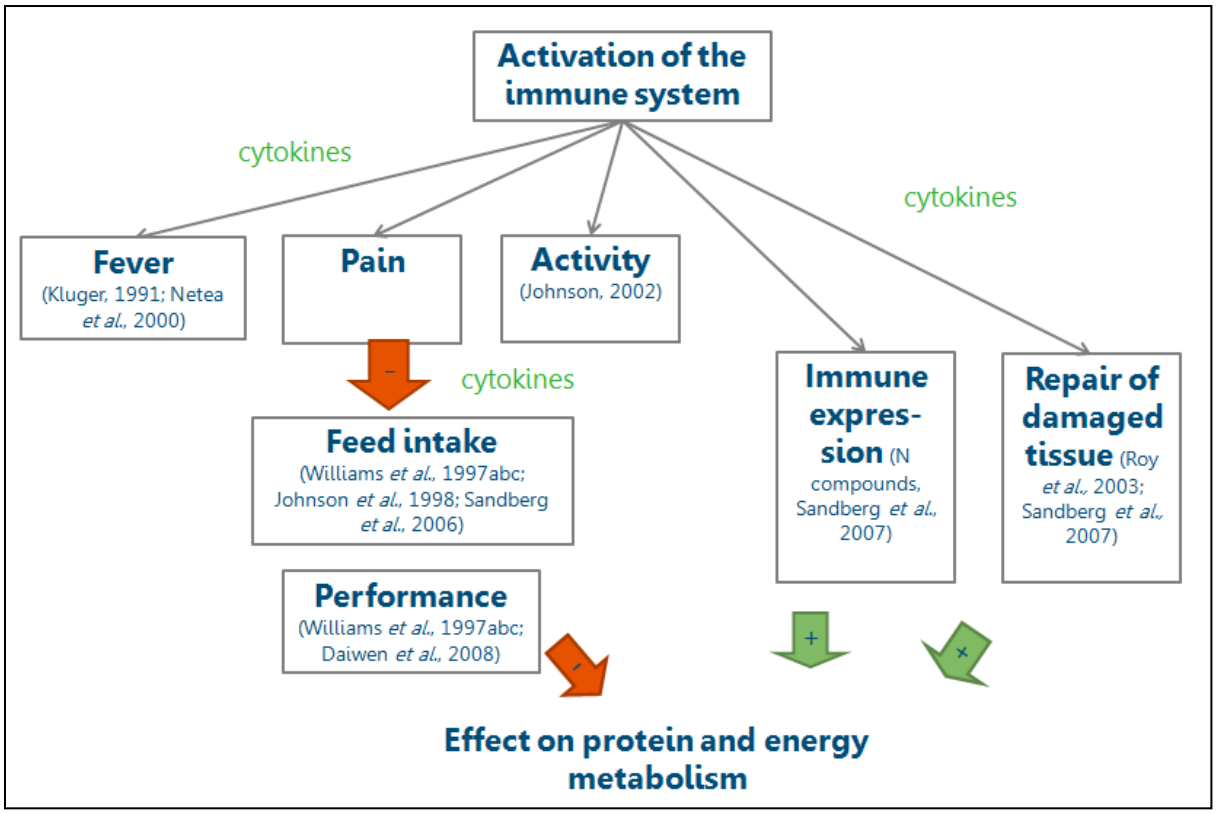


Figure 1.8 The effect of immune system activation on protein and energy metabolism.

The AA composition of APP differs, however, largely from that of muscle protein (Reeds *et al.*, 1994), and from commercial diets, which are formulated mainly to enhance muscle protein deposition. As a consequence, there can be an imbalance in AA available for body protein deposition during immune system activation, leading to increased oxidation of AA and increased loss of N via the urine (Reeds *et al.*, 1994). The calculations of Reeds *et al.* (1994), however, are based on a typical APP response in humans after uncomplicated surgery, and the AA composition of human APP, and the mean of bovine, porcine and ovine skeletal muscle. To my knowledge, calculations on the estimated increase in quantitative AA requirements for APP synthesis during an APP response in pigs after immune system activation are absent. Moreover, quantitative information about the effect of immune system activation on the requirements for AA, and moreover on the optimal dietary AA profile, *i.e.* the ratios between AA, is lacking, and measurements on changes in responses of multiple AA to immune system activation are largely absent.

Nutritional modulation of the immune system

There is increasing evidence that the dietary protein or AA supply can affect the inflammatory response during immune system activation, by impairing, maintaining, or

improving immune function (Grimble *et al.*, 1992; Jahoor *et al.*, 1999; Li *et al.*, 1999; Grimble, 2001; Adams, 2006; Li *et al.*, 2007; Le Floc'h *et al.*, 2008; Le Floc'h *et al.*, 2009; Calder and Yaqoob, 2012). Besides dietary deficiencies in energy (Fagbemi *et al.*, 1990), protein and AA, dietary deficiencies in vitamins, minerals, and fatty acids can impair immune functioning (Calder and Yaqoob, 2012). In this paragraph emphasis is on the detrimental effects of a deficient dietary AA supply on the immune system, as well as on beneficial effects of supplementary AA on the immune system.

Sulphur-containing AA (Met and Cys)

Live *E. coli* infected rats had increased glutathione synthesis rates in liver, spleen, large intestine, and lung, ranging between 1100% to 1450% and muscle and heart by approximately 180% compared to pair-fed controls (Malmezat *et al.*, 2000). Glutathione is present in cells as thiol-reduced (GSH) and disulfide-oxidized (GSSG) forms (DeLeve and Kaplowitz, 1991). The ratio between GSH and GSSG is the most important regulator of the redox potential (Roth, 2007). In addition, this ratio plays an important role in inflammation by initiating inflammatory cytokines through nuclear factor-κB, and by regulating cell proliferation and apoptosis (Roth *et al.*, 2002; Roth, 2007). Labelled Cys incorporation into glutathione was higher in spleen and kidneys of *E. coli* infected rats than pair-fed controls (Malmezat *et al.*, 1998). In addition, Cys incorporation into protein in spleen, lung, and in plasma proteins without albumin, was higher in *E. coli* infected rats than pair-fed controls (Malmezat *et al.*, 1998). Cys supplementation to a protein deficient diet increased liver weight, which was impaired in the protein deficient fed group, and increased hepatic glutathione concentrations following an intraperitoneal injection of pro-inflammatory cytokine TNF-α in rats (Grimble *et al.*, 1992). These findings indicate that Cys is important for GSH production, and that both Cys and GSH are important for antioxidant defences, by acting as scavengers of reactive oxygen species. Furthermore, pigs fed a low protein diet were not able to maintain homeostasis in red blood cell and mucosal GSH concentration or synthesis rate when subjected to inflammation by *s.c.* challenge with turpentine (Jahoor *et al.*, 1995). A study of Litvak *et al.* (2013a) revealed that after LPS administration, plasma albumin concentration and fractional synthesis rate were lower in pigs fed a diet deficient in Met and Cys, compared to a dietary supply that met the requirement for Met and Cys. Furthermore, Cys supplementation to *E. coli* infected rats was proven to be beneficial, as indicated by a reduced urinary nitrogen excretion and muscle weight loss (Breuillé *et al.*, 2006). Feeding high protein diets with a deficient Met content to rats increased the number of worm eggs in the colon following an infection with *Nippostrongylus brasiliensis* larvea, indicating a reduced resistance against parasites, but did not affect systemic Ig, mast and goblet cells, and eosinophil numbers (Sakkas *et al.*, 2012). Other products of sulphur AA are homocysteine and Tau, which can modulate the immune system, especially inflammation (Grimble, 2006). Grimble (2006) suggests that homocysteine stimulates monocyte activity, and increases the interactions between T lymphocytes, monocytes, and endothelium *in vitro*. Tau has

antioxidant properties, regulates the release of pro-inflammatory cytokines, and Tau deficiency in animals decreased the number of lymphocytes, increased monocytes, and impaired phagocytosis as reviewed by Grimble (2006). These studies indicate that dietary supplementation of Cys and or Met can be beneficial for immune functioning, whereas a deficiency can impair immune function.

Tryptophan

Trp deficiency in rats (Gershoff *et al.*, 1968; Kenney *et al.*, 1970) and mice (Qiu *et al.*, 2011) reduced antibody titres, and antibody titres linearly increased with increasing Trp supply, suggesting that Trp is an important mediator for maintaining immunoglobulin concentrations.

During immune system activation the kynurenine pathway is up-regulated, particularly by IFN-γ produced *e.g.* by dendritic cells, macrophages, eosinophills and endothelial cells, and is associated with an increased catabolism of Trp into kynurenine by IDO (Chen and Guillemin, 2009; de la Fuente *et al.*, 2012). IDO is expressed on cells in many tissues, and is induced by signals from the immune system (Mellor and Munn, 2004). Another rate limiting enzyme in Trp catabolism is hepatic Trp 2,3-dioxygenase (TDO), which, in contrast to IDO, is induced by Trp and metabolic steroids, and thus highly specific for the substrate tryptophan (Moffett and Namboodiri, 2003). Trp catabolism by IDO activity has a suppressive effect on the immune system, mainly by down-regulating the T cell and inflammatory response (Fallarino *et al.*, 2006; de la Fuente *et al.*, 2012; Mandi and Vécsei, 2012). More specifically, IDO supresses T cell proliferation and stimulates T cell apoptosis through the production of kynurenines, including kynurenine and quinolinic acid, in combination with dramatically reducing the supply of Trp in local cells (Fallarino *et al.*, 2006). Reducing the local supply of Trp could be a defence mechanism against pathogens (Moffett and Namboodiri, 2003). *In vitro*, IDO activity inhibits replication of certain bacteria and viruses which are sensitive to Trp depletion, although this mechanism remains unclear *in vivo* (Moffett and Namboodiri, 2003; Mellor and Munn, 2004). When deprived of Trp, T cells stop cell division, whereas an excess of Trp can reverse IDO mediated suppression of T cells (Mellor and Munn, 2004). Besides controlling parasite proliferation by local Trp depletion, IDO functions as a mechanism for immune tolerance. IDO expressed on dendritic cells stimulate the maturation of immature T cells into regulatory T cells, which play an important role in immunological self-tolerance, by controlling harmful self-reactive T cells (Wing and Sakaguchi, 2010). In this way over-activity of the immune system is prevented. In addition, IDO has antioxidant activity by consuming superoxide anions (Hayaishi, 1996), a free radical associated with cell damage. Other Trp metabolites that are modulators of the immune system include quinolinate, by being anti-inflammatory and inducing apoptosis, and kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilate, by supressing T cell proliferation, and inducing apoptosis in T cells and monocytes, and picolinic acid, by coactivating macrophages (Moffett and Namboodiri, 2003). Thus, Trp metabolism through the kynurenine pathway is suggested to be an important

regulator of the innate and adaptive immune responses. In complete Freund's adjuvant (CFA) challenged pigs, for instance, IDO activity in lung increases (Le Floc'h *et al.*, 2008). IDO activity in lungs and heart, and lung weight was, however, greater in CFA challenged pigs fed a deficient Trp diet than in CFA challenged pigs fed additional Trp (Le Floc'h *et al.*, 2008). As IDO is less substrate specific for Trp than TDO, and induced by cells of the immune system, the increased IDO activity observed in pigs fed a deficient Trp diet, together with the increased lung weight indicate a more severe immune response than in pigs fed an adequate Trp diet. In line, plasma haptoglobin concentrations in CFA challenged pigs fed a deficient Trp diet were still elevated at day 7 and 9 post-challenge compared to healthy pair-fed controls, whereas pigs fed an adequate Trp diet had similar plasma haptoglobin concentrations from day 7 onwards compared to healthy pair-fed controls (Le Floc'h *et al.*, 2008). These findings indicate that additional Trp helps to preserve the immune response to CFA, with less severe increases in lung weight and plasma haptoglobin concentrations.

BCAA (Leu, Ile, and Val)

In chickens, a diet deficient in BCAA *i.e.* at 50 or 16% of the recommended requirement value, was associated with a lower lymphoid organ weight and tended to reduce antibody titres, *i.e.* total haemagglutinin titre, against sheep red blood cells, compared to pair-fed controls (Konashi *et al.*, 2000). In that study, BCAA deficiency had the greatest potential to modulate the adaptive immune response in chickens, compared to a dietary deficiency in Met + Cys, Phe plus Tyr, Arg plus Lys, or other AA including Gly, Ser, His, Thr, and Trp. *In vitro* studies indicate that BCAA are essential for lymphocytes to synthesize protein, RNA and DNA and to proliferate after immune system activation, and that a deficient dietary supply of BCAA impairs killer-cell activity, reduces antibody titres, and increases the susceptibility to pathogens (Calder, 2006). In contrast, an excess of Leu, in combination with low levels of Val and Ile, can have detrimental effects by decreasing the humoral response to an i.m. challenge with keyhole limpet hemocyanin in weanling pigs (Gatnau *et al.*, 1995). Furthermore, BCAA, especially Leu, increase albumin synthesis in rat primary hepatocytes in a dose-dependent manner, through a key molecule mammalian target of rapamycin that triggers protein synthesis (Ijichi *et al.*, 2003).

Other essential and conditionally essential AA

It has recently been considered that Arg, Gln, Glu, Gly, Pro, Tau and Cys can become conditionally essential during periods of stress (*e.g.*, heat stress, burns, and infection) (Wu, 2013). "A 'conditionally essential' nutrient is a physiologically indispensable compound that is nutritionally nonessential for normal subjects, but is required in the diet by certain sick individuals because they have lost the capacity to synthesize it at an adequate rate" (Chipponi *et al.*, 1982). Arg, Gln and Tau are succesfully supplemented to improve clinical outcome in surgery and critically ill patients (Calder, 2007b). By

increasing the supply of AA required for the synthesis of mucins, *i.e.* Thr, Ser, Pro, and Cys, to rats with dextran sulfate sodium induced colitis, the number of mucin-containing goblet cells in the colon increased, mucin synthesis increased, and gut microbiota were promoted (Faure *et al.*, 2006). The latter authors suggested that supplementation of Thr, Ser, Pro, and Cys assist in colonic protection and mucosal healing. In piglets, a deficient Thr diet was associated with a lower villus height and crypt width in the ileum than in pair-fed control piglets receiving an adequate Thr diet (Hamard *et al.*, 2007). The addition of Thr to the diet of pigs increased serum IgG concentrations in response to ovalbumin (Wang *et al.*, 2006a) or to *s.c.* challenge with swine fever attenuated vaccine or bovine serum albumin (Li *et al.*, 1999). Arg is known as an important modulator of the immune system (Wu and Morris, 1998; Popovic *et al.*, 2007; Ruth and Field, 2013). Arg metabolism plays an important role in supressing activated T lymphocytes, by controlling myeloid suppressor cells, which activate the arginase-1 and NO synthase-2 pathways (Bronte and Zanovello, 2005). In addition, T lymphocytes depend on Arg for proliferation, antigen recognition, and the development of memory (Popovic *et al.*, 2007). Arg is the only precursor of NO, which is a key immunomodulator and is highly required by neutrophils during the innate response of an infection (Calder and Yaqoob, 2012; Ruth and Field, 2013). Induced by pro-inflammatory cytokines, including IFN-γ, IL-1, TNF-α, but also by the endotoxin LPS, macrophages express nitric oxide synthase-2, which converts Arg into NO and citrulline (Bredt and Snyder, 1994; Bronte and Zanovello, 2005). In turn, NO enhances cytotoxic and antimicrobial activity against pathogens and tumour cells (Macmicking *et al.*, 1997). In addition, NO regulates the vascular system by being a major endogenous vasodilator, and by inhibiting platelet aggregation and adhesion (Bredt and Snyder, 1994). Dietary Arg supplementation improves wound healing (Wu *et al.*, 2000; Stechmiller *et al.*, 2005). In addition, dietary Arg supplementation increased antibody production against *Salmonella*, suppressed the increase in serum CRP, IFN-γ and IL-12 concentrations, and down-regulated mRNA expression of TNF-α after an experimentally induced *Salmonella enterica* infection in weaned pigs (Chen *et al.*, 2012). The latter authors suggested that Arg supplementation has a protective effect by preventing overproduction of inflammatory cytokines. In addition, dietary Arg supplementation enhanced immune function in aged mice as indicated by increased delayed-type hypersensitivity response as measured by ear thickness to dinitrofluorobenzene, and increased popliteal lymph node weights in response to sheep red blood cells in aged mice (Lewis and Langkamp-Henken, 2000). In contrast, in the non-supplemented group both responses were impaired and associated with an age-dependent impairment of the immune system (Lewis and Langkamp-Henken, 2000). In addition, intravenous (*i.v.*) Arg infusion diminished intestinal damage in a neonatal piglet model of necrotizing enterocolitis (Di Lorenzo *et al.*, 1995). Nevertheless, Arg supplementation during inflammation can be both beneficial and harmful (Roth, 2007). Dietary Gln supplementation to rats with dextran sulfate sodium-induced colitis did not affect gut associated lymphocyte populations, however, a lower water content in feces was observed compared to dextran sulfate sodium induced

control rats, indicative for an improved barrier function in the colon (Vicario *et al.*, 2007). In pigs, dietary Gln supplementation increased or maintained villus height compared to control, which was indicative for preventing enterocyte destruction (Wu *et al.*, 1996). Gly supplementation to a low protein diet led to a higher serum APP as indicated by increased serum α-1-acid glycoprotein concentrations in response to a TNF-α injection in rats (Grimble *et al.*, 1992). In addition, dietary Gly prevents mortality, prevented inflammation and injury in the lung, by downregulating chloride channels on Kupffer cells in response to *i.v.* LPS administration (Wheeler *et al.*, 2000).

In conclusion, the dietary supply of (semi)-essential AA play an impotant role in host resistance, and are able to modulate immune functioning by supporting or, in case of AA deficiencies or extreme surplus, by impairing the immune system.

Methods for studying amino acid requirements

AA requirements are typically determined by varying the dietary supply of the first limiting AA, and measuring the response in protein metabolism, *e.g.* in N retention, BW gain, plasma AA or urea concentration (Pencharz and Ball, 2003). In growing pigs, separate groups are usually assigned to specific AA supply levels, and requirement values are derived from nonlinear regression analysis of measured responses (Batterham *et al.*, 1990; Rao and McCracken, 1990; Bikker *et al.*, 1994; Susenbeth *et al.*, 1994; Coma *et al.*, 1995). Kim *et al.* (1983) introduced the indicator AA oxidation (IAAO) technique, which has provided significant insight in the variation in AA requirements between individual pigs (Bertolo *et al.*, 2005; Moehn *et al.*, 2008). Yet, its application requires isotope infusion, mass spectrometry equipment, and steady-state conditions, hampering its application under meal-fed conditions of two to three daily feedings. Therefore, a technique was developed to estimate a quantitative change in the requirement of a limiting AA for protein deposition of individual meal fed pigs. Secondly, a technique was developed to measure changes in responses of multiple AA simultaneously to immune system activation. Changes in AA metabolism, *e.g.* an increased protein synthesis rate, can occur without concomitant changes in plasma AA concentrations or pool size, as AA concentrations can be maintained when fluxes from protein intake, breakdown and synthesis of body protein, and oxidation of AA are changing (Waterlow, 2006). Yet, changes in plasma AA concentrations have been used previously as a measure to assess effects of immune system activation on AA metabolism (Maes *et al.*, 1993; Melchior *et al.*, 2004; Melchior *et al.*, 2005; Le Floc'h *et al.*, 2006).

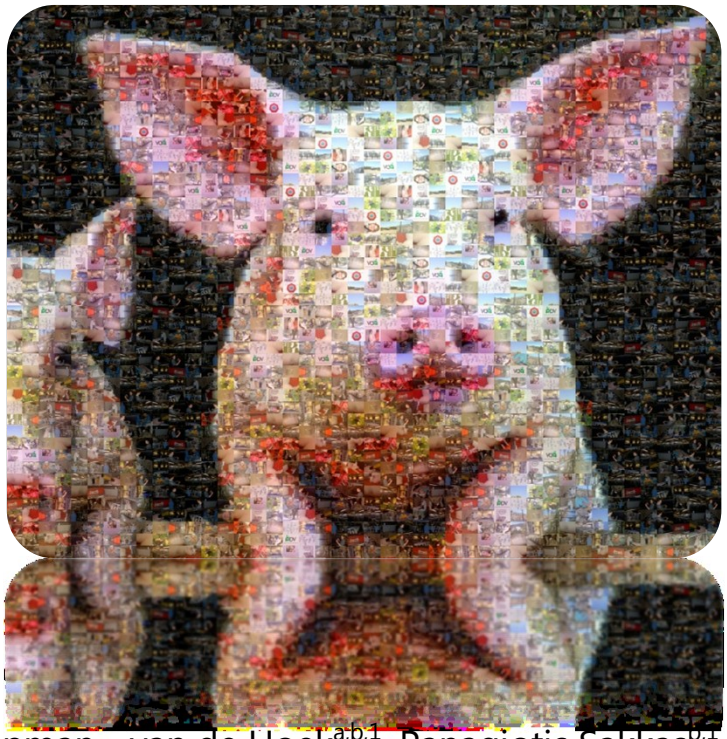
Aims and thesis outline

The main objective of this thesis is to quantify the effects of health status on AA requirements for body protein deposition and for immune system functioning of growing pigs. This information allows feed manufacturers to optimize pig diets by adjusting to variation in health status, and thereby contributes to further improving pig performance.

Firstly, the health status web as a concept for classification of the health status of growing-finishing pig farms was developed, based on data recorded in current commercial practice (**Chapter 2**). Techniques were developed for measuring AA requirements in individual pigs. A non-invasive dose-response technique was developed to quantify the requirement for Lys, and possibly for other AA in individual growing-pigs (**Chapter 3**). In addition, the isotope dilution technique was developed to provide insight in simultaneous changes in the metabolism of multiple AA as affected by differences in dietary supply of protein and AA. Next, an experiment was performed to determine the effects of health status and dietary deficiency of Met + Cys, Thr and Trp on N retention and AA metabolism in growing pigs, which were selected from two farms with a different health status (**Chapter 4**). A pilot study was performed to select an appropriate model for immune system activation. To quantify the effects of immune system activation and dietary protein supply on N retention and AA metabolism in growing pigs, a final study was conducted in growing pigs challenged with CFA as a model to activate the immune system (**Chapter 5**). In the General Discussion (**Chapter 6)**, the findings from Chapter 2 to 5 and pilot studies are discussed to provide quantitative information about the effect of health status on AA requirements for immune system functioning and for body protein deposition. Moreover, general conclusions and recommendations for future research are provided.

Chapter 2

A novel scoring system for the classification of the health status of growing-finishing pig farms



Esther Kampman - van de Hoek[a,b,1], Panagiotis Sakkas[b,1], Walter J. J. Gerrits[b], Hetty van Beers-Schreurs[c], Carola M. C. van der Peet-Schwering[a], Joost J. G. C. van den Borne[b], and Alfons J. M. Jansman[a]

[1]These authors contributed equally to this work.

To be submitted

[a]WUR Livestock Research, Wageningen, The Netherlands
[b]Animal Nutrition Group, Wageningen University, Wageningen, The Netherlands
[c]SDa, Autoriteit Diergeneesmiddelen, Utrecht, The Netherlands

Abstract

The aim of the current study was to develop a concept for classification of the health status of growing-finishing pig farms, based on commonly recorded data. Six traits were incorporated into a health status web, being average daily gain (ADG), energy conversion ratio (ECR), mortality, incidence of pleuritis, and percentage rejected lungs and livers at slaughter over a period of one year. Performance data from 1074 and 783 Dutch pig farms, and abattoir data of 50208 and 47426 farm deliveries to slaughterhouses, acquired over 2011 and 2012 respectively, were used as a representative sample for the Dutch growing-finishing pig population to calculate the 25th and 75th percentiles of each trait for each year. For each individual farm, a score was calculated per trait by inter- and extrapolation using the 25th and 75th percentiles from the Dutch growing-finishing pig population as reference. The farm score was defined as the mean score over the six traits. A farm was classified as follows: for a farm score between 50 and 62.5: suboptimal health; for a farm score between 62.5 and 87.5: conventional health; for a farm score between 87.5 and 100: high health.

To evaluate the health status web concept, two datasets were compiled: dataset 1 with individual farm data of 179 farms over the year 2011, and dataset 2 with individual farm data of 70 farms over both 2011, a subset of dataset 1, and 2012. In dataset 1, 13 farms were characterized as high health, 159 farms as conventional and seven farms as having a low health status. Farm scores were higher ($P < 0.001$) for gilt-boar farms than for gilt-barrow farms, urging the need for adjusting the 25th and 75th percentiles of each trait in the health status web according to farm subpopulations, *e.g.* according to sex, rather than to the farm population as a whole. Furthermore, it was concluded that the performance and abattoir data used to calculate the 25th and 75th percentiles of each trait change in time and therefore must be updated yearly. Dataset 2 revealed consistent farm scores across years, indicating that the farm score is farm specific and that the health status web is a valuable concept to characterize growing-finishing pig farms on the basis of their health status.

Keywords: Farm health status; growing pigs; performance; abattoir data.

Introduction

Animal health can be defined as the absence of disease as determined by clinical examinations in combination with various diagnostic tests *e.g.* for parasites or pathogenic micro-organisms (Petersen *et al.*, 2004). Clinical infections affecting pigs can be associated with high morbidity and mortality. Pigs, however, are continually exposed to a range of pathogens that can also cause varying degrees of nonspecific, subclinical disease (Cromwell, 2002). Subclinical infections are also problematic because they are difficult to detect by clinical examinations (Stark, 2000) and hamper the animal's overall growth performance (Le Floc'h *et al.*, 2004). It is very difficult to quantify and manage the impact of subclinical infections at farm level, especially when the nature of the pathogens is unknown (Clapperton *et al.*, 2005b).

Subclinical and clinical infections which activate the immune system can be bacterial, viral or parasitic in nature. They induce an inflammatory status through an activation of the immune system, which in turn does not allow the animal to achieve its full growth potential and compromises its well-being (Humphrey, 2008). This is evident by the fact that commercially reared pigs commonly fail to achieve their genetic potential for growth and efficiency (Holck *et al.*, 1998; Gabler and Spurlock, 2008). At the same time there is large variability in performance between farms which cannot be attributed to genotype and nutrition; *e.g.* 20% of Dutch growing-finishing pig farms have an average daily gain (ADG) of less than 762 g/day whereas another 20% of farms have and ADG of more than 830 g/day (AgroVision; 2012). It is suggested that activation of the immune system adversely affects performance, by inducing anorexia (Kyriazakis and Doelsch-Wilson, 2009) and by altering the energy expenditure and metabolism of amino acids (AA) (Reeds and Jahoor, 2001).

Current estimates for the nutrient requirements of pigs are based on experiments which have been largely performed under good sanitary conditions. Over the last decade it has been attempted to redefine these requirements, especially in relation to the demand for specific AA in inflammatory conditions (Li *et al.*, 1999; Le Floc'h *et al.*, 2008; Le Floc'h *et al.*, 2009; Wu, 2009; Rakhshandeh *et al.*, 2010). The need to redefine nutrient requirements is increased by the ban of in feed antibiotics as part of the routine management for prophylaxis and for promotion of growth performance, due to concerns about the emergence and development of antimicrobial resistance (Aarestrup *et al.*, 2008). Moreover, effects of subclinical disease might be more prevalent currently, as it has been proposed that selection for high lean meat deposition has resulted in pigs that are more susceptible to immunological stress (Frank *et al.*, 2005; Merlot *et al.*, 2012).

Classifying the health of pig herds is pivotal in applying targeted nutrition strategies and the implementation of husbandry measures related to the management of the herd, its housing and the environment of these farms with the aim to improve farm productivity. For the application of targeted nutritional strategies it is necessary to be able to easily identify farms that suffer from unsanitary conditions which induce

penalties in the efficiency of production. Currently, defining the health status of a farm requires assessment of clinical signs, post-mortem examination of dead pigs or those that are culled, vaccination and antibiotic use, slaughter-check examinations, and detection of specific pathogen occurrence through serology (Dewey and Straw, 2006). Over the last years, screening for concentrations of acute phase proteins (APP) in blood for defining the health status of farms and/or individual animals has attained considerable interest (Petersen *et al.*, 2004; Carroll *et al.*, 2004; Cray *et al.*, 2009). These assessments, however, are time consuming and costly, as they are not readily available. Moreover, serological tests may be indicative for the health status of individual pigs, but may be less indicative for the health status of a farm. In addition, blood concentrations of APP are affected by age and time after immune system activation (Gutiérrez *et al.*, 2009a), their response to subclinical problems may be short lived (days or weeks), and mainly reflect a short term health status. Although it is acknowledged that for monitoring the health status of individual farms the above mentioned assessments and diagnostic tests can be useful to improve the status and the productivity of a particular farm, the presented concept can be of use to adapt general nutritional or management strategies to suit the needs for particular groups of farms sharing a common health status. Therefore, a system for classification of farms should be employed, that incorporates traits which are registered, are readily available and are collectively related to (sub)clinical disease.

The aim of the present study was to develop a system that allows the classification of the health status of growing-finishing pig farms.

Materials and methods

Traits included in the scoring system

In a series of brainstorming sessions among specialists involved in the pig production sector, six traits were chosen on the basis of their relevance to the occurrence of clinical and subclinical infections at farm level and the availability of related relevant data from different sources. The proposed scoring system, hereafter referred to as health status web, incorporates six traits which are divided into two classes, being performance data and abattoir data, the latter being related to pathological deviations of specific organs in the slaughterhouse.

One of the hallmarks of clinical and subclinical infections is the induction of penalties in the production performance of pig farms. Pathogenic challenges disturb physiological processes through the production of cytokines. Activation of the innate immune system upon pathogen invasion, results in secretion of the cytokines interleukin-1, interleukin-6, tumour necrosis factor α, and interferon-γ by activated macrophages (Le Floc'h *et al.*, 2004). The latter induce inappetence and sickness behaviour (Johnson, 1998; Buchanan and Johnson, 2007) and as a result reduce nutrients available for protein deposition. In addition, they can inhibit nutrient absorption, increase metabolic rate, and alter nutrient utilization in a tissue-specific

manner (Johnson, 2002). Growth efficiency is negatively affected as nutrients and in particular AA are repartitioned away from body protein deposition towards tissues and cells involved in inflammatory and immune responses (Kyriazakis and Houdijk, 2006). Metabolic drains imposed by infection are related to the repair of damaged tissues, to the synthesis of lymphocytes, recruitment of new monocytes from the bone marrow, and synthesis of various proteinaceous molecules such as immunoglobulins, cytokines and APP (Colditz, 2002; Calder, 2006; Li *et al.*, 2007; Sandberg *et al.*, 2007; Wu, 2009). The repartitioning assists in delivering nutrients towards the immune system (Klasing, 1988; Colditz, 2002; Obled, 2003). Various studies have been performed involving deteriorated sanitary housing conditions (Williams *et al.*, 1997a,b,c; Le Floc'h *et al.*, 2009; Renaudeau, 2009; Le Floc'h *et al.*, 2010), administration of infectious pathogens (Greiner *et al.*, 2000; Escobar *et al.*, 2004; Davis *et al.*, 2010) or non-infectious immunogens such as lipopolysaccharide (LPS) (Van Heugten *et al.*, 1996; Webel *et al.*, 1997; Chen *et al.*, 2008), illustrating the adverse effects of immune system activation on feed intake, growth and efficiency of feed utilization. Recent meta-analysis studies associate the impact of infections on various aspects of production efficiency (Montagne *et al.*, 2010; Kipper *et al.*, 2011; Pastorelli *et al.*, 2012). Consequently, it is expected that the occurrence of (sub)clinical disease is reflected in traits related to the production performance of farms. In addition, it is easy to perceive that higher prevalence of infections is expected to increase mortality in the herd, as more animals are expected to develop clinical signs of disease. In the proposed scoring system we incorporated the traits ADG (g/day), energy conversion ratio (ECR; EW/kg ADG, where EW = dietary net energy intake (MJ; CVB, 1996) / 8.8 MJ), and mortality (%) during the starter, grower and finisher phase. The ADG and ECR were standardized at a body weight (BW) range of 25 - 112 kg. In the Netherlands, data on these traits are registered by farmers on a voluntary basis using farm management programs Pigmanager and FARM and are summarized yearly by AgroVision (2012).

Similarly, the health status of a farm is reflected on abnormalities detected in the slaughter line. Organized carcass inspection has long been recognized as a tool in assessing the health status of the herd (Willeberg *et al.*, 1984; Mousing *et al.*, 1990, Elbers, 1992). Since the 1990s results of individual inspection of all pigs delivered for slaughter are recorded in the Netherlands. Regulation (EC) No 854/2004 of the European Parliament was introduced as part of the 'EU Hygiene Package' which sets specific rules for the organization of official inspection controls on meat derived from pigs intended for human consumption (Hill, 2013). All abattoirs in the Netherlands are legally enforced to use the Dutch inspection procedure developed by the National Inspection Service for Livestock and Meat in the early 1990s (PVV, 2006). Pathological inspection results include lesions of livers, skin, legs, lungs and occurrence of pleuritis (van Wagenberg *et al.*, 2010). Respiratory infections are recognized as one of the most serious disease problems in pigs and their importance continue to increase with intensification of pig production (Sørensen *et al.*, 2006). They result in substantial economic losses due to poor growth performance, reduced feed efficiency and higher medication costs and have an adverse effect on pig welfare (Sørensen *et al.*, 2006).

Often respiratory infections do not show clinical signs of disease making abattoir examinations of thoracic organs important for evaluation of the health status of the herd (Andreasen *et al.*, 2001). Catarrhal bronchopneumonia and pleuritis are the most frequent lung lesions recorded on abattoir inspections with prevalence varying, depending on the country and the lung lesion scoring system that is used (Wilson *et al.*, 1986; Enøe *et al.*, 2002; Leneveu, 2005; Fraile *et al.*, 2010; Meyns *et al.*, 2011). The cause of respiratory diseases and the development of lung lesions is multifactorial and complex in nature, resulting from the interaction of multiple infectious agents such as bacteria, viruses, environmental conditions and host factors (Fablet *et al.*, 2012; Merialdi *et al.*, 2012). In pigs, the most important micro-organisms responsible for disease are *Mycoplasma hyopneumoniae, Pasteurella multocida, Actinobacillus pleuropneumoniae,* swine influenza viruses and porcine reproductive and respiratory syndrome virus (PRRSV) (Choi *et al.*, 2003; Sørensen *et al.*, 2006; Fablet *et al.*, 2012). Pathological inspections in abattoirs estimate the incidence of pneumonia lesions and chronic pleuritis. Lungs are visually inspected in abattoirs in the Netherlands and are scored as abnormal or rejected depending on the degree of macroscopic alterations. In addition, the percentage of lungs which are affected by pleuritis is recorded. Liver abnormalities can be caused by many systemic diseases which cause congestion and inflammatory cell infiltration. However, the most common one is parasitism, in particular with *Ascaris suum* larvae (van Wagenberg *et al.*, 2010; Vlaminck *et al.*, 2014), which adversely affects production performance of growing pigs (Hale *et al.*, 1985). Inspected livers are classified on the basis of the degree of pathological deformation due to *Ascaris suum* infection. A liver has minor lesions if it has one or two white spots on the front side, indicative for inflammatory tissue due to migration of larvae through the liver. When a liver has three or more white spots, it is rejected, and consequently declared unfit for human and animal consumption (van Wagenberg *et al.*, 2010). In the scoring system we developed, the occurrence of pulmonary changes (percentage of rejected lungs in the slaughter line), pleuritis (percentage of pigs showing abnormalities in the slaughter line due to pleuritis) and liver abnormalities (percentage of rejected livers in the slaughter line), were taken into account. The aforementioned abattoir data from Dutch growing-finishing pig farms over the years 2011, and 2012 were provided by the Vion Food Group (unpublished results).

Parameterization of the scoring system

The six underlying farm traits of the Dutch growing-finishing pig population, obtained from AgroVision (performance data) and Vion Food Group (abattoir data) were used to calculate the 25th and 75th percentiles, *i.e.* the value of a trait below which 25% or 75% of the observations in the Dutch growing-finishing pig population were found. For example, the 25th percentile for ADG indicates that 25% of the Dutch farms have an ADG equal to or less than 767 g/day. The 75th percentile for ADG indicates that 75% of the Dutch farms have an ADG equal to or less than 821 g/day (Table 2.1). Performance data from AgroVision included data of 1074 and 783 Dutch growing-finishing pig

farms over 2011 and 2012 respectively. The abattoir data from Vion Food Group included data of 50208 batches and 47426 batches, *i.e.* number of farm deliveries to the slaughterhouses, acquired over 2011 and 2012, respectively. The performance data were presented in five classes with an equal number of farms per class, ranking performance with an average value per class. The 25th and 75th percentiles of the performance traits ADG, and ECR, and for mortality (%) were calculated from these data using a normal distribution function:

$$f(x,\mu,\sigma) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-\mu)^2}{2\sigma^2}} \quad [1]$$

where x is the value for each trait, μ is the mean of the distribution and σ is the standard deviation (SD).

Table 2.1 Performance and abattoir data of a Dutch population of growing-finishing pig farms used to calculate percentiles and subsequent farm scores to characterize farms as having a suboptimal, conventional or high health status in the health status web.

Trait	50th percentile	25th percentile[1]	75th percentile[1]	Reference
ADG, g/day	794	767	821	AgroVision, 2011
	791	764	818	AgroVision, 2012
ECR, EW/kg[2]	2.83	2.95	2.73	AgroVision, 2011
	2.80	2.91	2.70	AgroVision, 2012
Mortality, %	2.4	3.1	1.7	AgroVision, 2011
	2.4	3.1	1.7	AgroVision, 2012
Pleuritis, %	4.7	19.1	2.0	Vion Food Group, 2011
	4.9	19.7	2.0	Vion Food Group, 2012
Lung, % rejected	3.6	8.7	1.4	Vion Food Group, 2011
	3.5	9.2	1.1	Vion Food Group, 2012
Liver, % rejected	1.7	3.6	0.0	Vion Food Group, 2011
	1.4	3.4	0.0	Vion Food Group, 2012

Abbreviations used: ADG, average daily gain; ECR, energy conversion ratio.
[1]The 25th and 75th percentiles of the performance traits ADG, and ECR, and for mortality (%) were calculated from data using the normal distribution function [1] (n = 1074 and 783 for 2011 and 2012, respectively). The 25th and 75th percentiles of the abattoir traits concerning the incidence of pleuritis, lung- and liver abnormalities were calculated from data providing the cumulative percentage of all batches of pigs recorded (n = 50208 and 47426 for 2011 and 2012, respectively).
[2]Energy conversion ratio (ECR) as energy intake (EW) per kg ADG, where EW = net energy (NE) in MJ per kg diet divided by 8.8 MJ (CVB, 1996).

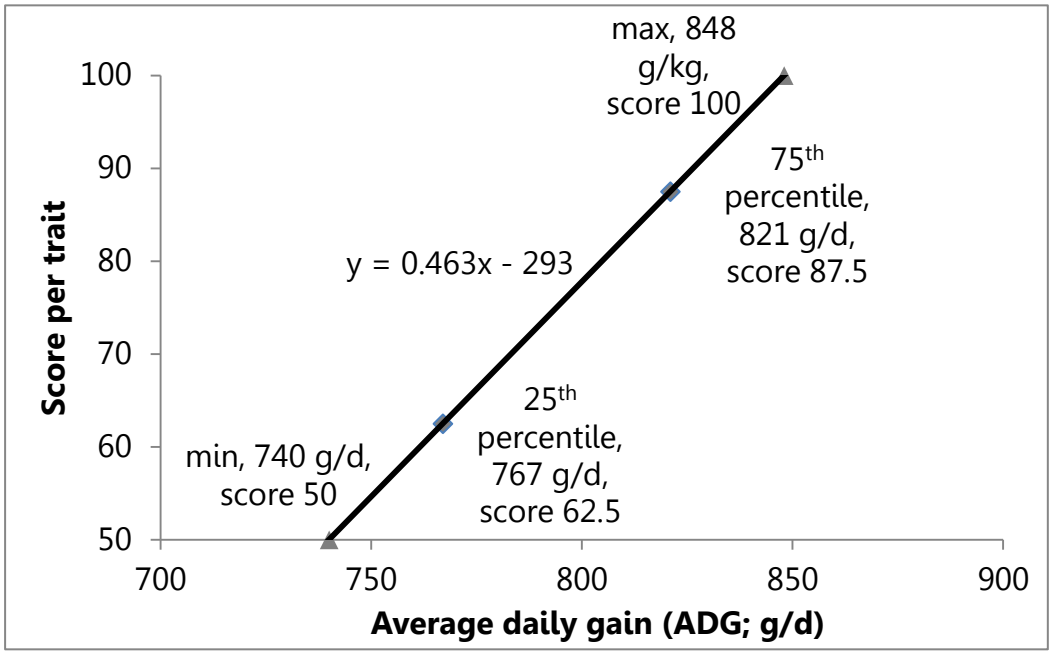


Figure 2.1 Graphical representation of a linear equation used to obtain a score per trait (y) from the value of one of the six traits of a particular farm (x), for example average daily gain (ADG; g/day), using the 25th and 75th percentiles of each trait derived from a Dutch population of growing-finishing pig farms obtained from AgroVision (performance data) and Vion Food Group (abattoir data). For each trait, a minimum (min) score of 50 and a maximum (max) score of 100 was established, corresponding with an ADG of 740 and 848 g/day, respectively.

Concerning the abattoir data, the percentage of pigs showing pleuritis, lung- and liver abnormalities at slaughter were not normally distributed. Therefore the 25th and 75th percentiles of these traits were calculated from data on these deviations on the cumulative percentage of batches of pigs slaughtered in classes of 10%. For each individual farm, a score was calculated per trait by inter- and extrapolation using the 25th and 75th percentiles from the Dutch growing-finishing pig population as reference (Figure 2.1). A trait score was set between a value of 50 (minimum) and 100 (maximum). The mean score of the six traits of a particular farm is referred to as the farm score. The standard deviation over the values of the scores for the six traits of each farm is a measure for the uniformity of the farm score. Finally, a farm was classified based on the farm score as follows: for farm scores between 50 and 62.5: suboptimal health; for farm scores between 62.5 and 87.5: conventional health; for farm scores between 87.5 and 100: high health.

Two datasets were compiled, dataset 1 with individual farm data of 179 farms over the year 2011 to characterize farms on the basis of their health status and to examine the effect of sex, *i.e.* gilt-barrow or gilt-boar, and genetic line on the farm score and the

uniformity of the farm score. Dataset 2 included individual farm data of 70 farms over both 2011 and 2012 and was used to evaluate the consistency in farm scores over years.

Statistical methods

Using dataset 1, the effect of health status classification group on the uniformity of the farm score was analysed by ANOVA using proc GLM in SAS (SAS Inst. Inc., Cary, NC, USA). When main effects were significant, *P*-values for differences of the Least squares means were used to compare the three health status classification groups. Similarly, the effect of sex, *i.e.* gilt-barrow or gilt-boar, or genetic line on the farm score and the uniformity of the farm score were analysed by ANOVA. In addition, the effect of sex on the six traits, ADG, ECR, mortality, incidence of pleuritis, and lung- and liver abnormalities, was analysed by ANOVA. Genetic lines were classified in 12 categories, including Hypor · Mix (*i.e.* mix of different lines, n = 5), Pietrain (n = 21), T20 · Pietrain (n = 26), T20 · Talent (n = 19), T20 · Tempo (n = 30), T20 · Mix (n = 8), T30 · Mix (n = 8), T40 · Pietrain (n = 2), T50 · Mix (n = 6), Talent (n = 8), Tempo (n = 27), "Other", including non-specified (n = 19).

Using dataset 2, it was tested whether the farm score was consistent over the years 2011 and 2012. Therefore, a correlation analysis between the farm score or farm uniformity over 2011 and 2012 was performed in SAS (SAS Inst. Inc., Cary, NC, USA). A paired t-test on farm score and on its uniformity was performed to test whether the mean difference in farm score between 2011 and 2012 was not statistically different from zero, which indicates that a representative sample of farms of the Dutch population of growing-finishing pig farms was used.

Results

The data on the six traits from a total of 179 growing-finishing pig farms over 2011 and from a total of 70 growing-finishing pig farms over both 2011 and 2012 are displayed in Table 2.2. By using the health status web we classified the 179 farms according to their health status. A graphical representation of the scores of two farms are displayed in Figure 2.2 and Figure 2.3. Of the 179 farms in dataset 1, 13 farms were characterized as high health, 159 farms as conventional and seven farms as having a low health status (Table 2.3). The uniformity of the farm score was higher (P = 0.02) in the high health status group than in the conventional health status group, but similar to that of the suboptimal health status group.

Table 2.2 Performance and abattoir data of 179 growing-finishing pig farms over the year 2011 (dataset 1) used to characterize farms according to their health status, and of 70 farms over the year 2012a (dataset 2) used to compare the characterization of farms between 2011 and 2012.

Trait	Mean	SD	Min	Max
Dataset 1 (n = 179)				
ADG, g/day	802	45.5	678	925
ECR, EW/kg[2]	2.81	0.133	2.48	3.16
Mortality, %	2.2	0.94	0.5	7.1
Pleuritis, %	7.9	6.24	0	34.7
Lung, % rejected	6.4	4.35	0	20
Liver, % rejected	2.3	2.99	0	22.3
Dataset 2 (n = 70)[1]				
ADG, g/day	802	53.4	671	907
ECR, EW/kg[2]	2.77	0.143	2.50	3.16
Mortality, %	2.1	0.89	0.5	5.0
Pleuritis, %	9.5	6.84	1.0	39.4
Lung, % rejected	8.5	5.81	0.7	33.9
Liver, % rejected	1.9	1.85	0.3	12.4

Abbreviations used: ADG, average daily gain; ECR, energy conversion ratio; SD, standard deviation.
[1]Dataset 2 includes the 70 farms that are also part of dataset 1.
[2]Energy conversion ratio (ECR) as energy intake (EW) per kg ADG, where EW = net energy (NE) in MJ per kg diet divided by 8.8 MJ (CVB, 1996).

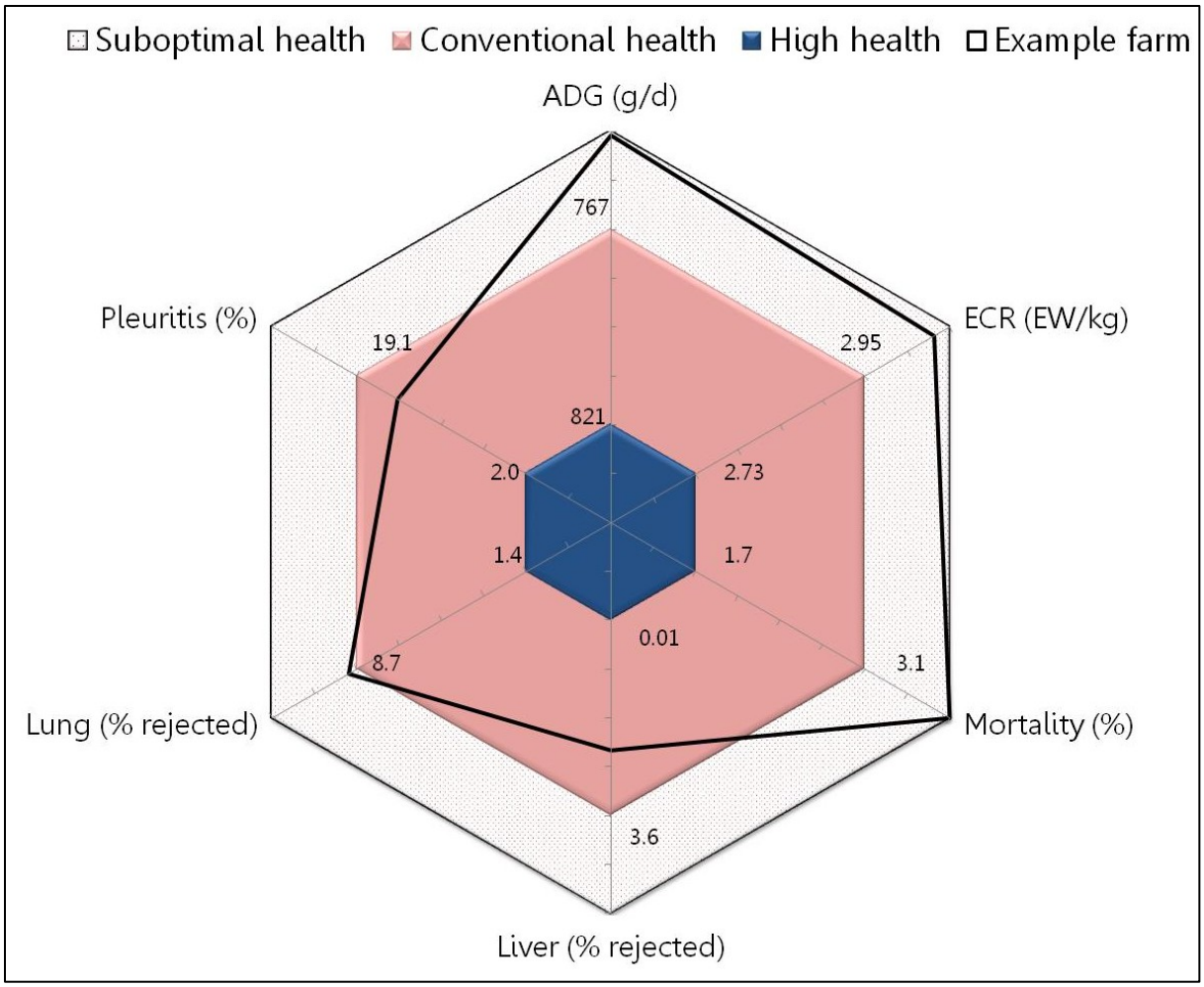


Figure 2.2 Graphical representation of the health status web of a farm characterized as having a low health status with farm score 59 and uniformity of the farm score 9.3. The farm score is referred to as the mean score of the six traits of a particular farm and characterizes farms as having a suboptimal (farm score between 50 and 62.5), conventional (farm score between 62.5 and 87.5) or high health status (farm score between 87.5 and 100). The uniformity of the farm score is calculated as the standard deviation of scores for the six traits for each farm. The 25th and 75th percentiles of the Dutch growing-finishing pig population are displayed for each trait.

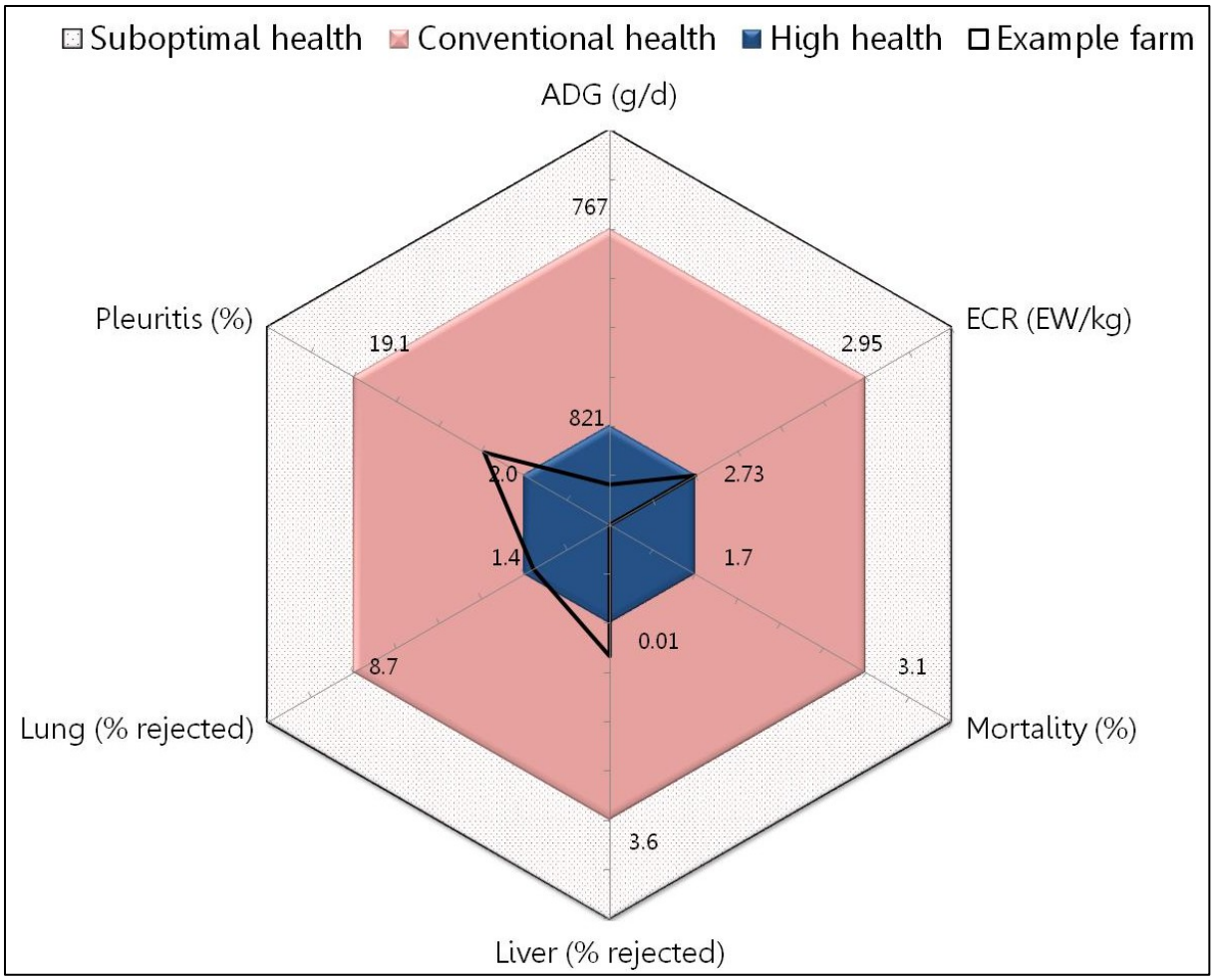


Figure 2.3 Graphical representation of the health status web of a farm characterized as having a high health status with farm score 89 and uniformity of the farm score 7.0. The farm score is referred to as the mean score of the six traits of a particular farm and characterizes farms as having a suboptimal (farm score between 50 and 62.5), conventional (farm score between 62.5 and 87.5) or high health status (farm score between 87.5 and 100). The uniformity of the farm score is calculated as the standard deviation of scores for the six traits for each farm. The 25th and 75th percentiles of the Dutch growing-finishing pig population are displayed for each trait.

Table 2.3 Characterization of 179 farms over the year 2011 based on the six traits used in the health status web.

	Suboptimal health status	Conventional health status	High health status
	Farm score 50 to 62.5	Farm score >62.5 to <87.5	Farm score 87.5 to 100
Number of farms	7	159	13
Farm score	58	76	90
Uniformity of the farm score[1]	10.0[ab]	11.1[b]	8.2[a]

[a,b]Values without a common superscripts within a row differ ($P < 0.05$).
[1]The mean of the standard deviation (SD) of the score of the six traits of each farm in the health status web. A low SD is indicative for a high uniformity of the farm score *vice versa*.

Dataset 1 included one farm with only gilts, one farm with gilts, barrows and boars, 90 farms with gilts and barrows and 87 farms with gilts and boars. Because of the low number of farms, data of the two farms with gilts or gilts, barrows and boars were excluded from the analysis. Of the 90 farms with gilts and barrows, seven farms were categorized as having a suboptimal health status (8%), 81 with a conventional health status (90%), and two farms with a high health status (2%). Within the 87 farms with gilts and boars, no farms were categorized as having a suboptimal health status (0%), 76 with a conventional health status (87%), and 11 farms with a high health status (13%). The farm score was higher (P <0.001) in gilt-boar than in gilt-barrow farms (Table 2.4). The uniformity of the farm score was not affected by sex (Table 2.4). When analysing the six traits separately, ADG (P <0.001) was higher, and ECR (P <0.001), and the incidence of lung abnormalities (P = 0.04) were lower in gilt-boar than in gilt-barrow farms and incidence of pleuritis tended to be lower (P = 0.06). The incidence of liver abnormalities and mortality were similar between gilt-boar and gilt-barrow farms.

Table 2.4 Effect of sex (gilt-barrow or gilt-boar farm) on farm score over the year 2011 based on the six traits used in the health status web[1].

	Gilt-barrow, n = 90	Git-boar, n = 87
Farm score	74[a]	79[b]
Uniformity of the farm score[1]	10	11

[a,b]Values without a common superscripts within a row differ ($P < 0.001$).
[1]The mean standard deviation (SD) of the score of the six traits of each farm in the health status web. A low SD is indicative for a high uniformity of the farm score *vice versa*.

Of the 163 farms with a specified genetic line, the four largest classes were T20 · Tempo pigs with 30 farms of which one farm was categorized as a high health status farm and two as suboptimal, Tempo pigs with 27 farms of which two farms were categorized as a high health status farm and two as suboptimal, T20 · Pietrain with 26 farms of which one farm was categorized as a high health status farm and one as

suboptimal, and 21 Pietrain pigs of which four farms were categorized as a high health status farm. The genetic line neither affected farm score nor its uniformity (data not shown).

The farm score of 70 farms was compared between the year 2011 and 2012. The difference in the farm score of individual farms between 2012 and 2011 was not different from zero (P = 0.47) with a mean difference of 0.6 ± 0.86 (SE). The mean difference in uniformity of the farm score (2012 minus 2011) was -0.1 ± 0.41 (SE, P = 0.78). The farm scores over 2011 and 2012 were correlated (r = 0.64, P < 0.0001), as well as the uniformity of the farm score over 2011 and 2012 (r = 0.59, P < 0.0001; Figure 2.4). The farm score of most of the farms (50 out of 70, *i.e.* 71%) did not change with more than five points between 2011 and 2012 (Figure 2.5). Out of 70 farms, seven farms (10%) shifted from one health status category to another category, of which three farms had a major change in farm score, *i.e.* of more than 10 points between both years (Table 2.5), while uniformity of the farm score was not subject to major change over both years (data not shown).

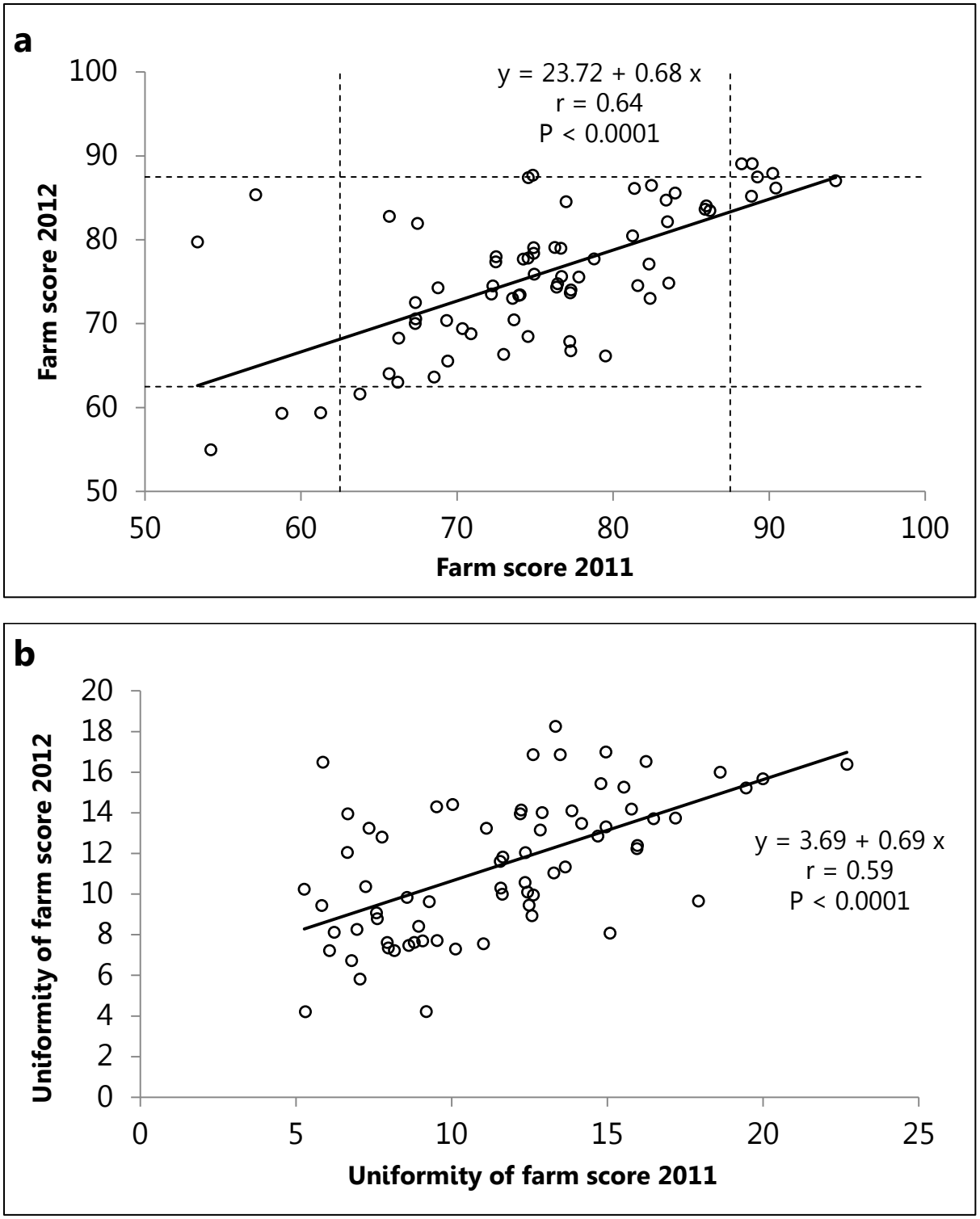


Figure 2.4 Correlation plot between farm score (a) or uniformity of the farm score (b) over the year 2011 and 2012 of 70 farms based on the six traits used in the health status web. Dashes lines in panel a refer to the boundary values for the farm score which characterizes farms as having a suboptimal (farm score between 50 and 62.5), conventional (farm score between 62.5 and 87.5) or high health status (farm score between 87.5 and 100).

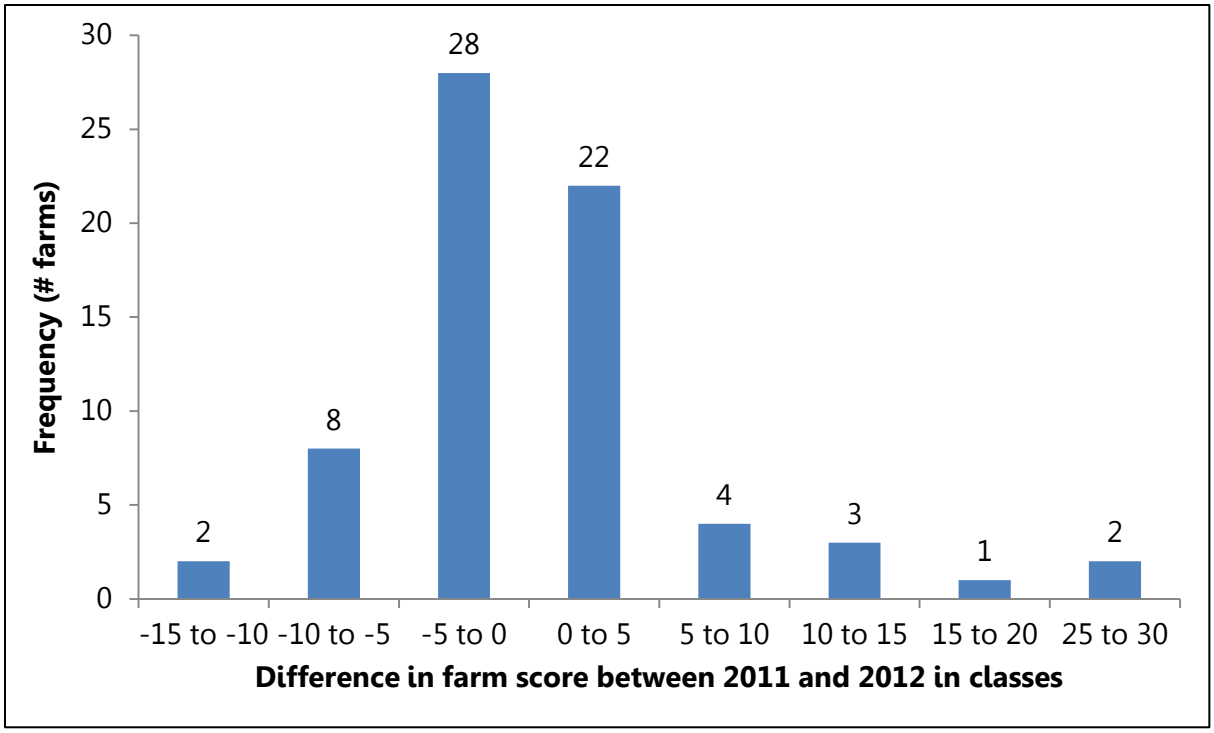


Figure 2.5 Frequency distribution of the difference in farm score (2012 minus 2011) of 70 farms based on the six traits used in the health status web.

Table 2.5 Number of farms shifting between health status categories from 2011 to 2012 out of 70 farms.

Suboptimal Health		Conventional health		High health	Difference in farm score per farm (2012 minus 2011)
2/70 (3%)	→				26; 28
	←	1/70 (1%)			-2
		1/70 (1%)	→		13
			←	3/70 (4%)	-4; -4; -7

Discussion

The aim of the present study was to develop a system that allows the classification of the health status of growing-finishing pig farms. This concept, that incorporates traits which are registered at farm level, readily available and are collectively related to (sub)clinical disease, can be used by farmers to monitor medium to long term changes in health status of their farm in relation to the pig population. In addition, it can be used by related industries to develop targeted strategies for improving efficiency of pig production. These may include farm-specific nutritional strategies, husbandry or breeding management strategies, *e.g.* genetic selection towards disease resistance which requires numerous traits to be monitored simultaneously (Bishop *et al.*, 2010). For feed companies, for instance, the health status web can be applied to categorize farms on the basis of their health status and in turn, formulate diets for groups of farms and feeding concepts to improve production performance. In addition, animal extension services may use the concept to identify low heath status farms and take appropriate management actions that improve farm health and productivity. With this concept it is also possible to study trends in farm health status in a certain population of farms compared to other populations of farms or compared to the country's pig farm population as a whole. The concept principally can also be applied to other livestock species, depending on currently available data on relevant traits.

Data on the performance traits ADG, ECR, and mortality, and the incidence of pleuritis and lung- or liver abnormalities at slaughter, as traits incorporated in the health status web, were selected based on the direct or indirect relation to the incidence of (sub)clinical disease and the availability of data, *i.e.* they are routinely recorded at farm level. The extent of antibiotic use at farm level could also be a meaningful trait to include in our health status web, however, no correlation between therapeutic antibiotic use and the performance of farms was observed in the data used for the development of the present health status web (data not shown) and in literature (Dolman *et al.*, 2012). In addition, data on therapeutic antibiotic use are not readily available yet, and on farm antibiotic use is subject to great variation over time. For example, in the Netherlands the annual antibiotic use has decreased by more than 60% in fattening pigs between 2008 and 2012 as a result of the implementation of a policy of the Dutch government to reduce antibiotic use in the animal production sector (Bondt *et al.*, 2012). Nevertheless, in the future, the extent of antibiotic use at farm level could also be a meaningful trait to incorporate in the concept of the health status web.

With regard to data obtained in abattoirs, it should be noticed that post mortem findings can vary substantially among meat inspectors and abattoirs as shown for *e.g.* the detection of liver abnormalities (Enøe *et al.*, 2003). In contrast, variation in the detection of pleuritis and liver abnormalities has been suggested to be small (Bonde *et al.*, 2010; Schleicher *et al.*, 2013). It has to be stated, however, that especially data on liver abnormalities as used in the current study should be interpreted with some caution. In the current study, information on the combination of the six traits is

believed to be a valuable measure for the characterization of farms on the basis of their health status.

Performance and abattoir data of a Dutch population of growing-finishing pig farms were used to create subsequent farm scores to characterize farms as having a suboptimal, conventional or high health status in the health status web. We acknowledge that the six traits can also be affected by other factors such as the genetic background, the ratio of boars : barrows : gilts, nutrition, housing and management. In fact, data of 179 farms used to develop the health status web, indicate that gilt-boar farms have on average a higher farm score than gilt-barrow farms. Boars are known to have a greater feed intake, ADG, and more favourable feed conversion ratio than barrows (van der Peet-Schwering *et al.*, 2012). The fact that the farm score is different between sexes urges the need for calculating the 25^{th} and 75^{th} percentiles of each trait in the health status web for subpopulations of farms rather than for the farm population as a whole. This could result in the development of health status webs per sex, *e.g.* specific for gilt-boar or gilt-barrow farms. Another issue in the health status web is the time period over which the data of the traits in the health status are determined. In our health status web for each of the six traits 25^{th} and 75^{th} percentiles and related boundary values for calculating a farm score per trait are calculated per year, based on data collected over a large population of farms over a period of one year. This is believed to be an appropriate time period, as seasonal changes can affect the incidence of diseases on farms (Maclachlan and Dubovi, 2011). Moreover, we are interested in the characterization of the long term health status of a farm, rather than in a characterization of the short term status that could be largely influenced by incidental health problems.

It is well recognized that farmers will strive to improve the health status of their farm, and particularly farms with major (sub)clinical problems can achieve a substantially different farm scores in adjacent years. Nonetheless, consistency of farm scores over years would demonstrate the validity of the concept. The farm scores of 2011 and 2012 were highly correlated. In line, the farm score of the vast majority of farms (50 out of 70, *i.e.* 71%) did not change by more than 5 points between 2011 and 2012. These findings demonstrate that the health status web is a farm specific and consistent concept to characterize farms on the basis of their health status. The mean difference in farm score between 2011 and 2012 was not statistically different from zero, indicative for the use of data of a representative sample of farms in both years, reflecting the Dutch population of growing-finishing pig farms.

In the present study, high health farms had a higher uniformity of the farm score than conventional health farms, and numerically improved uniformity compared to farms categorized as having a suboptimal health status. These data demonstrate that the uniformity of the farm score improved with increasing overall farm score and health status. Farms with a suboptimal health status are possibly more subject to short term incidences that may have a more severe effect on uniformity of the farm score than long term effects, which in turn are expected to be indicative for a farm specific long

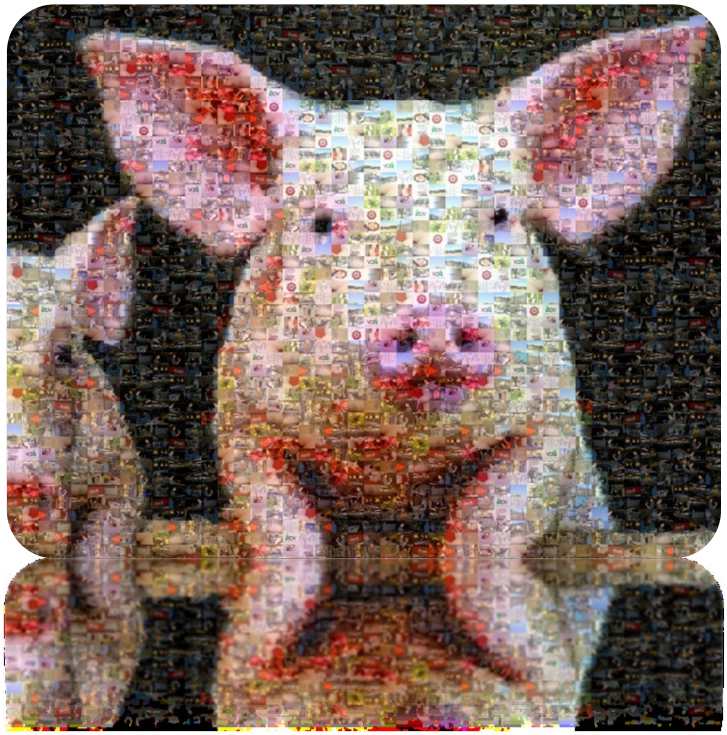
term health status. Furthermore, knowledge about the uniformity of a farm score with respect to the uniformity of farm scores within a population of farms can be important for making strategic decisions in order to improve the health status and productivity.

Conclusions

The health status web that incorporates ADG, ECR, mortality, the incidence of pleuritis and lung and liver abnormalities at slaughter is a farm specific and consistent concept to categorize farms with growing-finishing pigs on the basis of their health status, provided that the data of the six underlying farm traits of the growing-finishing population are updated each year. Farm scores were demonstrated to be consistent over years. The concept can be improved by developing sex or genotype specific webs per country. The concept can be used to characterize the health status of pig farms or other livestock species, as long as data of a substantial population of farms are available.

Chapter 3

A simple amino acid dose-response method to quantify amino acid requirements of individual meal-fed pigs



Esther Kampman - van de Hoek[ab], Walter J. J. Gerrits[b], Carola M. C. van der Peet-Schwering[a], Alfons J. M. Jansman[a], and Joost J. G. C. van den Borne[b]

Journal of Animal Science (2013) **91**, 4788-4796

[a]WUR Livestock Research, 8200 AB Lelystad, The Netherlands

[b]Animal Nutrition Group, Wageningen University, 6700 AH Wageningen, The Netherlands

Abstract

Two experiments were conducted to develop a simplified dose-response technique to estimate the Lys requirement of individual, meal-fed growing pigs. In Exp. 1, we studied adaptation processes that occur during such a dose-response study in meal-fed pigs, and in Exp. 2, we studied the accuracy of this simplified technique to estimate changes in Lys requirement estimates of pigs following changes in energy intake. In Exp. 1, the effect of the Lys supply strategy on the Lys requirement was assessed in 14 barrows fed an increasing (low to high, LH) or decreasing (high to low, HL) total Lys supply, with total Lys levels varying from 0.36 to 1.06 g/MJ DE in 7 equidistant steps of 4 days each. Urinary urea and ammonia excretion and whole body N turnover were measured. In Exp. 2, the accuracy of the dose-response technique to determine a shift in Lys requirement was assessed in 20 barrows fed at either 2.2 (low energy, LE) or 2.7 (high energy, HE) times the energy requirements for maintenance, with total Lys supply decreasing from 1.10 to 0.37 g Lys/MJ DE in 9 equidistant steps of 3 days each. In Exp. 1, a lower increment in protein synthesis, breakdown, and whole body N turnover with increasing dietary Lys supply was observed in LH-pigs than HL-pigs ($P < 0.01$) and the estimated Lys requirement was 0.06 g/MJ DE greater ($P = 0.01$) in LH-pigs than HL-pigs. These results indicated that pigs at a decreasing Lys supply strategy require less time for metabolic adaptation to a change in Lys supply than those at an increasing Lys supply. In Exp. 2, the estimated Lys requirement was 2.6 g/day greater ($P < 0.001$) in HE-pigs than LE-pigs. The variation in AA requirement estimates between individual pigs was low (4.9% in LH-pigs and 3.0% in HL-pigs in Exp. 1, and 8.1% in LE-pigs and 6.0% in HE-pigs in Exp. 2). The present studies indicated that a dose-response technique with a decreasing Lys supply in time and a step length of 3 days with urinary N excretion as response criteria provides a simple, accurate technique to quantitatively estimate a change in AA requirements of individual meal-fed pigs.

Keywords: amino acid requirement; individual variation; meal-fed pigs; metabolic adaptation, protein turnover; urinary nitrogen.

Introduction

Amino acid (AA) requirements are typically determined by varying the dietary supply of the first limiting AA and measuring the response in protein metabolism (Pencharz and Ball, 2003). In contrast to traditional methods (Bikker *et al.*, 1994; Coma *et al.*, 1995), Kim *et al.* (1983) introduced the indicator AA oxidation (IAAO) technique, which has provided considerable insight into the variation in AA requirements between individual pigs (Bertolo *et al.*, 2005; Moehn *et al.*, 2008). Yet, its application requires isotope infusion, mass spectrometry equipment, and steady-state conditions, hampering its application under meal-fed conditions of 2 to 3 daily feedings. A random order of AA supply levels has been applied to each subject in IAAO studies (Bertolo *et al.*, 2005; Moehn *et al.*, 2008), whereas others have applied an increased supply over time (Heger *et al.*, 2008, 2009).

Das and Waterlow (1974) found that 6 enzymes involved in the urea cycle adapted without lag time to a reduction in dietary protein level from 230 to 50 g/kg, whereas a lag time of 6 h was observed to adapt to an increase in dietary protein level from 50 to 230 g/kg. This indicates that the direction of change in protein or AA supply can influence the adaptation process to the change. We hypothesize that the rate of adaptation of protein metabolism to changes in AA supply is affected by the order in which graded AA levels are tested (an increasing or a decreasing supply strategy), and as a consequence, influences AA requirement estimates.

The aim of the present studies was to develop a simplified dose-response technique, with urinary response variables, to estimate the Lys requirement of individual, meal-fed growing pigs. The objective of Exp. 1 was to determine the effect of the Lys supply strategy (increasing *vs.* decreasing) on the rate of adaptation of protein turnover and on Lys requirement estimates of individual pigs. The objective of Exp. 2 was to assess the accuracy of the dose-response technique for determining a shift in AA requirements of individual pigs.

Materials and methods

The experiments were approved by the Animal Experimental Committees of Wageningen University (Exp. 1) and Wageningen UR Livestock Research (Exp. 2).

Animals and treatments

In Exp. 1, 14 barrows (York · Topigs 30 (Topigs, Helvoirt, The Netherlands)) with a BW of 27.1 ± 0.3 kg at the start of the study were individually housed in metabolism cages (1.5 · 0.6 m) at a room temperature of 22°C. Pigs were allocated, by equalizing mean BW between treatment groups and minimizing variation in BW among pigs within treatment groups, to either an increasing dietary Lys supply strategy (low to high, LH; n = 7) or a decreasing Lys supply strategy (high to low, HL; n = 7) with total Lys levels varying from 0.36 to 1.06 g/MJ DE (Figure 3.1). For a period of 12 days before the start of the study, pigs were adapted to housing conditions and experimental diets. For 9 d, pigs were fed a diet containing 0.72 g Lys/MJ DE followed by 3 d, in which they were

fed a diet with 0.36 (LH) or 1.06 g Lys/MJ DE (HL). Subsequently, a 28-days dose-response study was performed, in which Lys supply increased (LH) or decreased (HL) in 7 equidistant steps of 4 days each. The 7 Lys levels were created by mixing 2 basal diets (Table 3.1) with Lys levels of 0.36 and 1.06 g/MJ DE, respectively, in the appropriate ratios. The experimental diets were provided in mash form and mixed with water with a feed to water ratio of 1:3. Pigs were fed at 0730 and 1530 h in equal amounts at 2.5 times the energy requirements for maintenance (M; 458 kJ ME/(kg $BW^{0.75}$/day); ARC, 1981). In Exp. 1, feed allowance was adjusted on the first day of each Lys supply level (*i.e.*, every 4 d) based on BW at start of the dose-response period and an assumed BW gain of 450 g/day.

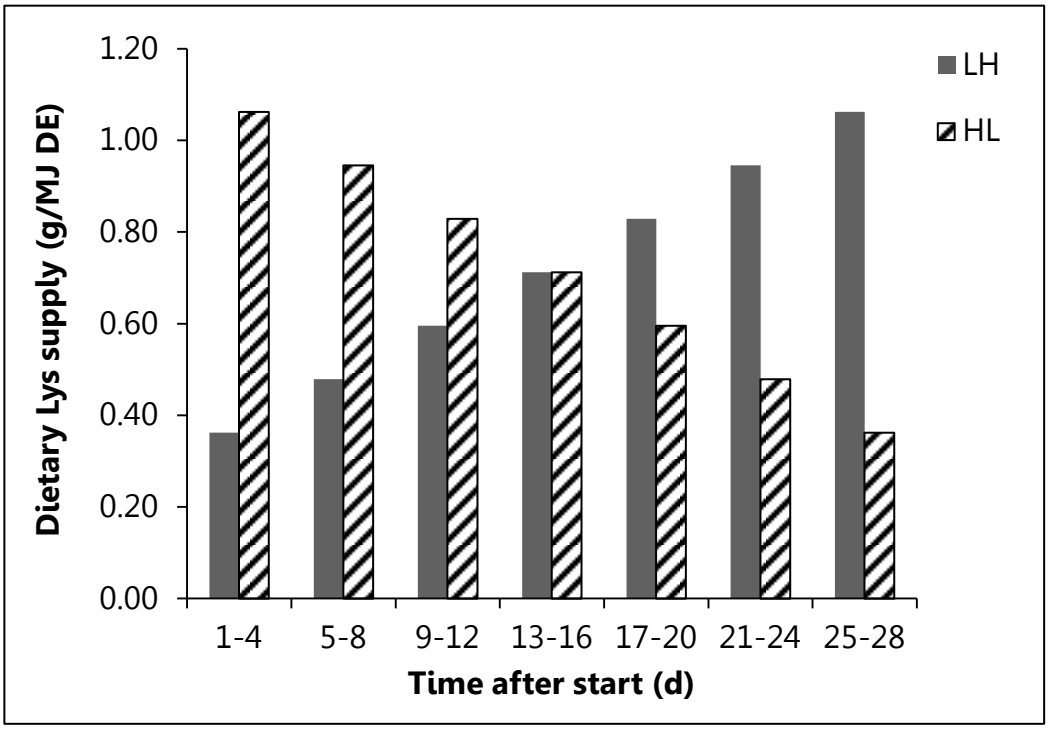


Figure 3.1 Changes in the total Lys supply during the experimental period for the increasing (low to high, LH) and decreasing (high to low, HL) Lys supply strategies (total Lys = 0.36 to 1.06 g/MJ DE) imposed on growing pigs in Exp. 1.

Table 3.1 Composition of low- and high-Lys basal diets fed to growing pigs (as-fed basis; Exp. 1)[1].

	Total Lys, g/MJ DE	
Item	**0.36**	**1.06**
Ingredient, g/kg		
Wheat	467.9	456.0
Barley	250.0	250.0
Wheat gluten meal	100.0	100.0
Sugar beet pulp	50.0	50.0
Soy protein concentrate	42.0	42.0
Soybean oil	30.0	30.0
Sucrose	20.0	20.0
Calcium carbonate	13.5	13.5
Monocalcium phosphate	10.5	10.5
Sodium carbonate	10.0	10.0
Vitamin and mineral premix[2]	5.0	5.0
L-Thr	0.9	0.9
L-Trp	0.2	0.2
L-Lys HCl	-	11.9
Calculated composition, g/kg[3]		
DM	884	886
NE[4], MJ/kg	10.0	10.0
DE[5], MJ/kg	14.0	14.1
CP	190	198
Crude ash	47	50
Crude fat	48	48
Crude fiber	34	34
AID[6] Lys	4.7	14.0
AID Met	2.6	2.6
AID Met + Cys	6.0	6.0
AID Thr	5.4	5.4
AID Trp	1.9	1.9
AID Ile	6.2	6.2
AID His	3.9	3.9
AID Leu	11.7	11.7
AID Val	5.8	5.8
Analyzed composition, g/kg		
DM	885	885
CP	187	194
Total Lys	5.1	15.0

[1]Seven diets were created by mixing 2 basal diets with 0.36 and 1.06 g/MJ DE.
[2]Premix provided per kilogram of diet: 5,000 IU of vitamin A; 1,000 IU of cholecalciferol; 7.5 IU of vitamin E; 0.4 mg of vitamin K; 3.5 mg of riboflavin; 5 mg of pantothenic acid; 20 mg of niacin amide; 15 µg of vitamin B_{12}; 200 mg of choline chloride; 80 mg of Fe as $FeSO_4 \cdot 7H_2O$; 20 mg of Cu as $CuSO_4 \cdot 5H_2O$; 73 mg of Zn as $ZnSO_4 \cdot H_2O$; 44 mg of Mn as MnO_2; 0.2 mg of Co as $CoSO_4 \cdot 7H_2O$; and 0.4 mg of I as KI; 0.06 mg Se as organic Se.
[3]Unless indicated otherwise.
[4]NE was calculated based on CVB (1996).
[5]DE was calculated based on CVB (1996) and Sauvant *et al.* (2004).
[6]AID = apparent ileal digestible.

In Exp. 2, 20 barrows (Dutch Landrace · York) with a BW of 31.5 ± 0.5 kg at the start of the experiment were individually housed in metabolism cages (1.3 · 1.3 m) at a room temperature ranging between 20 and 25°C. Pigs were allocated, by distributing littermates and equalizing mean BW between treatment groups and minimizing variation in BW among pigs within treatment groups, to 1 of 2 treatment groups receiving a dietary energy supply of 2.2 · M (low energy, LE) or 2.7 · M (high energy, HE). The HE treatment was created by providing additional maize starch to the dietary LE treatment (Table 3.2). For a period of 12 days before the start of the experiment, pigs were adapted to housing conditions and experimental diets. For 9 d, pigs were fed a diet with 0.74 g Lys/MJ DE followed by 3 d, in which they were fed a diet with 1.10 g Lys/MJ DE. During the first 5 days of the adaptation period, pigs were fed at an intermediate energy supply of 2.45 · M. Pigs were fed according to their treatment from 7 days before the start of the measurements onward. Subsequently, a 27-days dose-response study was performed, in which Lys supply decreased from 1.10 to 0.37 g Lys/MJ DE in 9 equidistant steps of 3 days each. The 9 Lys levels in the experimental diets were created by mixing 2 basal diets (Table 3.2) with Lys levels of 0.37 and 1.10 g/MJ DE, respectively, in the appropriate ratios. The experimental diets were provided in pelleted form and mixed with water with a feed to water ratio of 1:3. Pigs were fed at 0700, 1300, and 1900 h in equal amounts.

Table 3.2 Composition of low- and high-Lys basal diets fed at low- (LE) and high-energy (HE) supply to growing pigs (as-fed basis; Exp. 2)[1].

	LE		HE	
	Total Lys, g/kg		Total Lys, g/kg	
Item	5.1	15.2	5.1	15.5
Ingredient composition, g/kg				
Wheat	422.9	422.9	351.0	351.0
Barley	250.0	250.0	207.5	207.5
Wheat gluten meal	125.1	125.1	103.8	103.8
Sugar beet pulp	50.0	50.0	41.5	41.5
Soy protein concentrate	31.1	31.1	25.8	25.8
Soybean oil	30.0	30.0	24.9	24.9
Sucrose	20.0	20.0	16.6	16.6
Calcium carbonate	17.1	17.1	14.2	14.2
Maize starch	13.8	-	184.3	170.0
Monocalcium phosphate	13.8	13.8	11.5	11.5
Diamol	11.0	9.3	1.8	-
Salt	4.8	-	4.9	-
Potassium carbonate	3.8	3.6	5.4	5.2
Sodium carbonate	-	6.7	-	6.8
Vitamin and mineral premix[2]	2.4	2.4	2.0	2.0
L-Thr	2.1	2.1	1.8	1.8
DL-Met	1.0	1.0	0.8	0.8
L-Trp	0.6	0.6	0.5	0.5
L-Val	0.6	0.6	0.5	0.5
L-Lys HCl	-	13.8	1.3	15.6
Calculated nutrient composition[3], g/kg				
NE[4], MJ/kg	9.94	9.94	10.25	10.25
DE[5], MJ/kg	13.9	13.9	14.1	14.1
Crude ash	66	69	51	54
Crude fat	53	53	45	45
Crude fiber	32	32	27	27
AID[6] Lys	4.5	15.4	4.7	15.9
AID Met	3.8	3.8	3.2	3.2
AID Met + Cys	7.5	7.5	6.2	6.2
AID Thr	6.8	6.8	5.5	5.6
AID Trp	2.3	2.3	1.9	1.9
AID Ile	6.6	6.6	5.4	5.4
AID His	4.0	4.0	3.3	3.3
AID Leu	12.3	12.3	10.1	10.1
AID Val	8.0	8.0	6.4	6.4
Analyzed nutrient composition, g/kg				
DM	889	888	888	888
CP	194	202	162	176
Starch	408	391	484	465
Total Lys	5.1	15.2	5.1	15.5

[1]Nine diets were created for the LE and 9 for the HE treatment by mixing 2 diets 1 low- and one high-Lys.

[2]Premix provided per kilogram of LE or HE diet, respectively: 9,600 or 8,000 IU of vitamin A; 2,400 or 2,000 IU of cholecalciferol; 24 or 20 IU of vitamin E; 1.8 or 1.5 mg of vitamin K; 1.2 or 1

mg of thiamin; 4.8 or 4 mg of riboflavin; 14.4 or 12 mg of pantothenic acid; 24 or 20 mg of niacin; 24 or 20 µg of vitamin B_{12}; 0.24 or 0.20 mg of folate; 1.2 or 1.0 mg of vitamin B_6; 120or 100 mg of choline chloride; 120 or 100 mg of Fe as $FeSO_4$; 1.2 or 1.0 mg of Cu as $CuSO_4 \cdot 5H_2O$; 78 or 65 mg of Zn as ZnO; 36 or 30 mg of Mn as MnO; 0.18 or 0.15 mg of Co as $CoSO_4$; 0.90 or 0.75 mg of K as KI; and 0.36 or 0.30 mg of Se as Na-selenite.
[3]Unless indicated otherwise.
[4]NE was calculated based on CVB (1996).
[5]DE was calculated based on CVB (1996) and Sauvant *et al.* (2004).
[6]AID = apparent ileal digestible.

In both experiments, diets were formulated to be first limiting in Lys. The supply of other indispensable AA exceeded the estimated requirements for growing pigs of that BW (CVB, 1996; NRC, 1998). Pigs were weighed at arrival, and at the start and end of the dose-response period. In Exp. 2, pigs were weighed on the first day of each Lys level (*i.e.*, every 3 d) to adjust their feed allowance, which was based on BW and expected BW gain (calculated from the mean daily BW gain over the preceding 6 d).

Urine collection

Unpooled urine samples were collected quantitatively per 24 h over 4 subsequent days at each Lys level in Exp. 1 and over the last 2 days at each Lys level (day 2 and 3) in Exp. 2. Urine was collected via funnels, which were sprayed with an acetic acid buffer to prevent evaporation of NH_3, into buckets containing sulfuric acid (9 N) for conservation. Feces were collected using plastic bags (15 · 25 cm) attached around the anus of the pigs using a Velcro support system (Van Kleef *et al.*, 1994) and disposed directly after each feeding. Urine was collected after the morning feeding and stored at -20°C pending analysis.

Whole body nitrogen turnover

In Exp. 1, whole body N turnover was measured by the end-product method (Waterlow *et al.*, 1978). At 90 min after the morning feeding on day 3 of each Lys level, 300 mg ^{15}N-Gly (Isotec, Miamisburg, OH) was supplied with 100 g of feed, which was omitted from the morning feeding. The 48-h urine samples (of day 3 and 4 at each Lys level) were stored at -20°C pending measurement of ^{15}N enrichment in ammonia and urea. Background ^{15}N enrichment was determined in urine collected on day 2 at each Lys supply level.

Chemical analyses

In Exp. 1, N in urine of each of the subsequent 4 days of each Lys level was analyzed using the Kjeldahl method. Urine of day 3 and 4 of each Lys level was pooled and analyzed for urea (colorimetric method; Human, Wiesbaden, Germany) and ammonia (colorimetric method, Berthelot reaction). The ^{15}N enrichment was measured in urinary urea and ammonia after combustion of isolated urea and ammonia by isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany). For urea, samples were

deproteinized by adding 5 mL cold methanol and vortex-mixed and stored at -20°C for 1 h. Samples were centrifuged at 4,500 · *g* for 20 min at 4°C and the supernatant was passed over a cation exchange column (Dowex 50WX8-200; Sigma Aldrich, St. Louis, MO) to separate urea from other N-containing components in urine. The column was washed with 40 mL of distilled water, and the eluent was evaporated using a rotary evaporator. One milliliter of distilled water was added to the residue and this solution was transferred into a 1.5 mL micro centrifuge tube. The solution was freeze-dried and 100 µL of distilled water was added, mixed and transferred into a tin capsule. After evaporation of water at 40°C, the capsules were combusted and analyzed for ^{15}N enrichment in urea. For ammonia, isolation was performed by micro-diffusion (Conway, 1962). After 24 h, the N from ammonia in the sulfuric acid solution was harvested, freeze-dried, combusted in tin capsules, and analyzed for 15N enrichment. In Exp. 2, the concentration of urea plus ammonia in urine of day 2 and 3 of each Lys supply level was determined using a commercial enzymatic kit (UV method, R-Biopharm AG, Darmstadt, Germany). Diets were analyzed for N by the Kjeldahl method and crude protein was calculated (ISO, 2005a). Diets were analyzed for AA by HPLC after hydrolysis in hydrochloric acid (ISO, 2005b). In Exp. 2, starch content was determined enzymatically (NEN, 1992). All analyses were performed in duplicate.

Calculations

Urinary N excretion, calculated as the sum of N in urea and ammonia, was expressed relative to N intake. A reduction in urinary N excretion relative to N intake indicates an improved N utilization for protein deposition.

Whole body N turnover was calculated using the formula (Waterlow *et al.*, 1978):

$$Q_a \text{ or } Q_u = E_m \cdot do/e_m \quad [1]$$

in which Q_a represents the N flux (in mmol N/day) calculated from ammonia as end-product and Q_u represents the N flux with urea as end-product, E_m represents the rate of N excretion in the end-product (ammonia or urea, in mmol N/day), do represents the dose of ^{15}N-glycine (in mmol ^{15}N), and e_m represents the total amount of ^{15}N excreted, in excess of background, in the end-product (in ^{15}N mmol/day). The arithmetic average of Q_a and Q_u was calculated for estimating whole body N turnover, assuming that partitioning of tracer over the 2 precursor compartments is similar (Fern *et al.*, 1985). Turnover values were expressed in g/day. Whole body protein synthesis and breakdown were calculated assuming a steady-state in the AA pool over the 48-h period (Waterlow *et al.*, 1978; Waterlow, 2006):

$$Q = S + E = B + I \quad [2]$$

in which S is protein synthesis, E is urinary N excretion, B is protein breakdown, and I is N intake (all in g N/day).

Statistical analyses

The pig was considered as the experimental unit. A linear-plateau model, as described by Koops and Grossman (1993), was fitted to urinary N excretion data or whole body N turnover data across Lys supply levels for each pig:

$$Y = a + b \cdot X - b \cdot s \cdot \ln (1 + e^{(X - c)/s}) \quad [3]$$

in which Y is urinary N excretion in urea and ammonia (in % of N intake) or whole body N turnover (in g/day), X represents the Lys supply (in g/MJ DE in Exp. 1, and in g/day in Exp. 2), a represents the predicted urinary N excretion or N turnover at zero Lys supply, b represents the predicted rate of change for each unit of increase in Lys supply in the linear phase, c represents the transition point between the 2 phases, *i.e.*, the estimated Lys requirement, and s is a smoothness parameter for transition between the 2 phases of the model that was fixed at 0.01, resulting in a sharp transition. The urinary N excretion (% of intake) at the plateau was calculated as a + b · c. In addition, a linear model was fitted to data from urinary N excretion or whole body N turnover across Lys levels for each pig:

$$Y = a \cdot + b \quad [4]$$

in which a represents the rate of change for each unit of increase in Lys supply and b the intercept, *i.e.*, urinary N excretion or N turnover at zero Lys supply.

The nonlinear least squares regression procedure (PROC NLIN; SAS Inst. Inc., Cary, NC) was used for fitting models [3] and [4] to the data for each pig. The goodness of fit of the linear-plateau model [3] was assessed by comparing the sum of squares error from the linear-plateau model with that from the linear model [4] in an F-test. Parameter estimates were accepted when the linear-plateau model [3] provided a better fit of data ($P < 0.10$) than the linear model [4]. If this was not the case, pigs were excluded from further analyses of N excretion data. Parameter estimates were analyzed by ANOVA with Lys supply strategy (Exp. 1) and energy supply strategy (Exp. 2) as fixed effects, followed by LSD tests. In Exp. 1 the effect of urinary collection day (day 1, 2, 3, or 4) on urinary N excretion in the Lys limiting phase (linear phase before the breakpoint) was analyzed by a mixed model with collection day nested within dietary Lys level as repeated measures. The fixed effects included collection day nested within dietary Lys level, supply strategy, dietary Lys level, and the interaction between supply strategy and dietary Lys level and the interaction between supply strategy and collection day. Effects were analyzed by pairwise comparisons using Tukey-Kramer adjustment. The covariance structure was chosen based on the lowest value for the Akaike and Bayesian information criteria. In all analyses, the normality of the distribution of studentized residuals was assessed by the Shapiro-Wilk statistic. In Exp. 1, the slope parameters for N excretion and N turnover were transformed (logarithm and tangent, respectively) to obtain normal distribution of residuals. All statistical procedures were conducted in SAS. Values are presented as means ± SEM, and effects were considered significant at $P \leq 0.05$.

Results

Increasing vs. *decreasing Lys supply (Exp. 1)*

All pigs remained healthy during the experiment and feed refusals did not occur. The BW did not differ between treatments and averaged 27.1 ± 0.3 kg at the start and 43.9 ± 0.4 kg at the end of the experiment (n = 14).

The linear-plateau model [3] described the relationship between Lys supply (g/MJ DE) and urinary N excretion (% of intake) better ($P < 0.10$) than the linear model [4] in 11 out of 14 pigs. The estimated Lys requirement was 0.06 g/MJ DE greater ($P = 0.01$) for LH-pigs than for HL-pigs (Table 3.3). The strategy of Lys supply did not affect the other parameter estimates (*i.e.*, intercept, slope, and plateau). The CV of the requirement estimate for Lys (g/MJ DE) was 4.9% for LH (n = 4) and 3.0% for HL (n = 7). The data and model estimates for individual pigs are presented in Supplemental Figure 3.1 and 3.2.

Table 3.3 Effect of dietary Lys supply strategy on the linear-plateau model parameter estimates describing the relationship between total Lys supply (g/MJ DE) and urinary N excretion (% of N intake) in growing pigs (27 to 44 kg BW; Exp. 1).

	Lys supply strategy[3]		
Item[1,2]	**LH**	**HL**	***P*-value**
n	4	7	
Intercept	64.0 ± 2.4	64.2 ± 1.1	0.90
Slope	-44.1 ± 3.3	-47.5 ± 1.2	0.22
Breakpoint	0.84 ± 0.02	0.78 ± 0.01	0.01
Plateau	27.3 ± 0.3	27.3 ± 0.5	0.96

[1]The intercept represents the urinary N excretion at zero Lys supply. The slope represents the increment in urinary N excretion (% of N intake/(g Lys/MJ DE)). The breakpoint represents the total Lys supply, at which the linear phase transits into the plateau phase (*i.e.*, Lys requirement). The plateau represents the minimal urinary N excretion.
[2]The slope was log-transformed to obtain normal distribution of residuals.
[3]LH (low to high) = increasing Lys supply strategy and HL (high to low) = decreasing Lys supply strategy, and total Lys ranged from 0.36 to 1.06 g Lys/MJ DE.

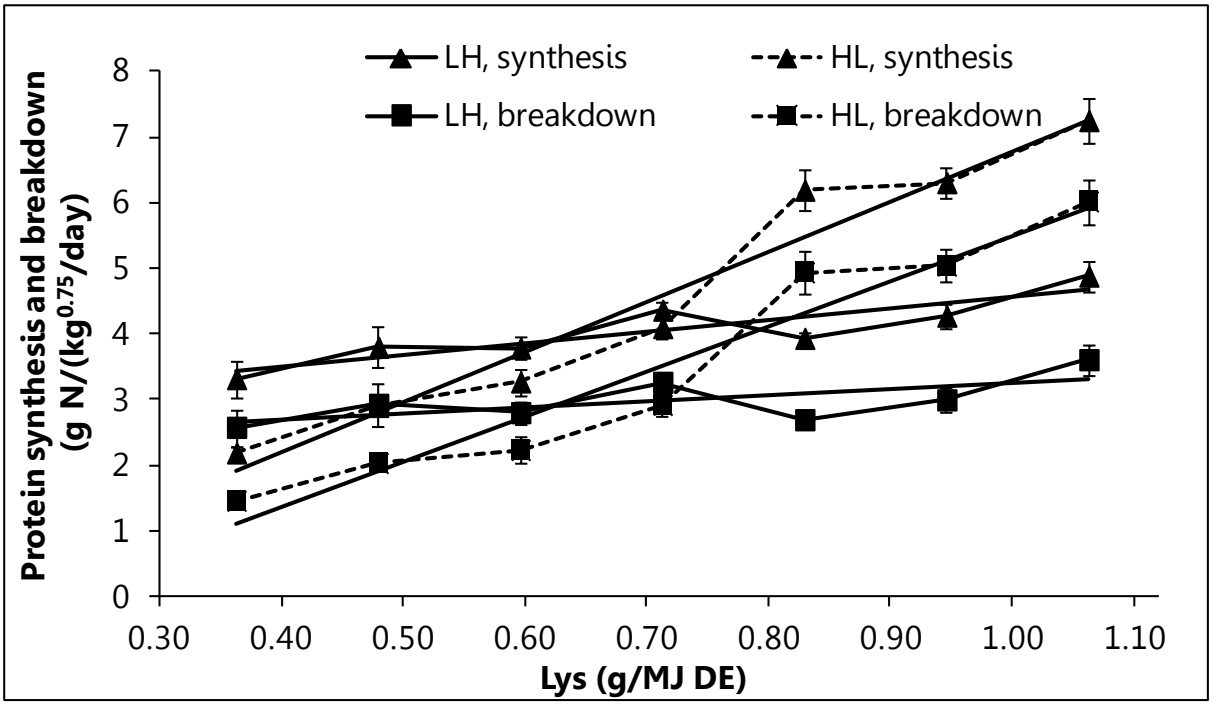


Figure 3.2 Effect of dietary Lys supply strategy on whole body protein synthesis and breakdown (g N/(kg$^{0.75}$/day)) in growing pigs fed various total Lys (g/MJ DE) in Exp. 1.

The linear model [4] described the relationship between Lys supply (g/MJ DE) and whole body N turnover (g N/(kg$^{0.75}$/day)) better than the linear-plateau model [3] in 13 out of 14 pigs, therefore, the linear model [4] was used. Whole body N turnover rate increased by 3.9 g N/(kg$^{0.75}$/day) per gram of increase in dietary Lys per MJ DE in HL pigs, resulting from an increase in synthesis (+5.2 g N/(kg$^{0.75}$/day)) and breakdown rates (+3.9 g N/(kg$^{0.75}$/day)). In contrast to HL pigs, the increase in whole body N turnover rate per gram of increase in dietary Lys/MJ DE was lower (P = 0.01) in LH pigs (+1.6 g N/(kg$^{0.75}$/day) because of a lower increase in synthesis (P < 0.01) and breakdown (P = 0.01) rates (+2.6 and +1.6 g N/(kg$^{0.75}$/day), respectively; Table 3.4). The estimated protein synthesis and breakdown rates per Lys level are presented in Figure 3.2. In the Lys limiting phase (linear phase before the breakpoint), urinary N excretion was different between the dietary total Lys levels (P < 0.001), but did not differ between collection days within each Lys level, and interactions between collection day and Lys supply strategy did not occur.

Table 3.4 Effect of dietary Lys supply strategy on the relationship between dietary Lys supply (g MJ DE) and whole body N turnover (g/kg$^{0.75}$/day) in the Lys limiting phase in growing pigs (27 to 44 kg; Exp. 1).

Item[1]	Lys supply strategy[2]		*P*-value
	LH	HL	
n	7	7	
Protein synthesis			
Intercept	2.4 ± 0.5	0.3 ± 0.2	< 0.01
Slope	2.6 ± 0.6	5.2 ± 0.4	< 0.01
Protein breakdown			
Intercept	2.0 ± 0.5	0.1 ± 0.2	< 0.01
Slope	1.6 ± 0.7	3.9 ± 0.4	0.01
Whole body N turnover			
Intercept	4.1 ± 0.5	2.2 ± 0.2	< 0.01
Slope	1.6 ± 0.5	3.9 ± 0.4	0.01

[1]The intercept represents whole body N turnover (g N/(kg$^{0.75}$/day)) at zero Lys supply. The slope represents the increment in whole body N turnover ((g N/kg$^{0.75}$/day)/(g Lys/MJ DE)).
[2]LH (low to high) = increasing Lys supply strategy and HL (high to low) = decreasing Lys supply strategy, and total Lys ranged from 0.36 to 1.06 g Lys/MJ DE.

Low vs. *high dietary energy supply (Exp. 2)*

One LE-pig was excluded from the experiment due to health problems and feed refusals. In 1 HE-pig, the data at a Lys level of 1.10 g/MJ DE (*i.e.*, the first 3 days of the experiment) were excluded due to feed refusals. The BW at the start of the experiment was similar between treatments (31.5 ± 0.5 kg; n = 19). At the end of the experiment, BW of LE-pigs (46.6 ± 1.0 kg) was lower ($P < 0.01$) than that of HE-pigs (52.5 ± 1.1 kg).

The linear-plateau model [3] described the relationship between Lys supply (g/day) and urinary N excretion (% of intake) better ($P < 0.10$) than the linear model [4] for all 19 pigs. The data and model estimates for individual pigs are presented in Supplemental Figure 3.3 and 3.4. The estimated Lys requirement was 2.6 g/day greater ($P < 0.001$) in HE-pigs than LE-pigs (Table 3.5) and the variation in estimated Lys requirement between individual pigs was numerically larger for LE (CV, 8.1%; n = 9) than for HE (CV, 6.0%; n = 10; Figure 3.3). An increase in dietary energy supply by 0.5 · M decreased ($P < 0.001$) N excretion at the plateau with 8.6 percentage points (Table 3.5).

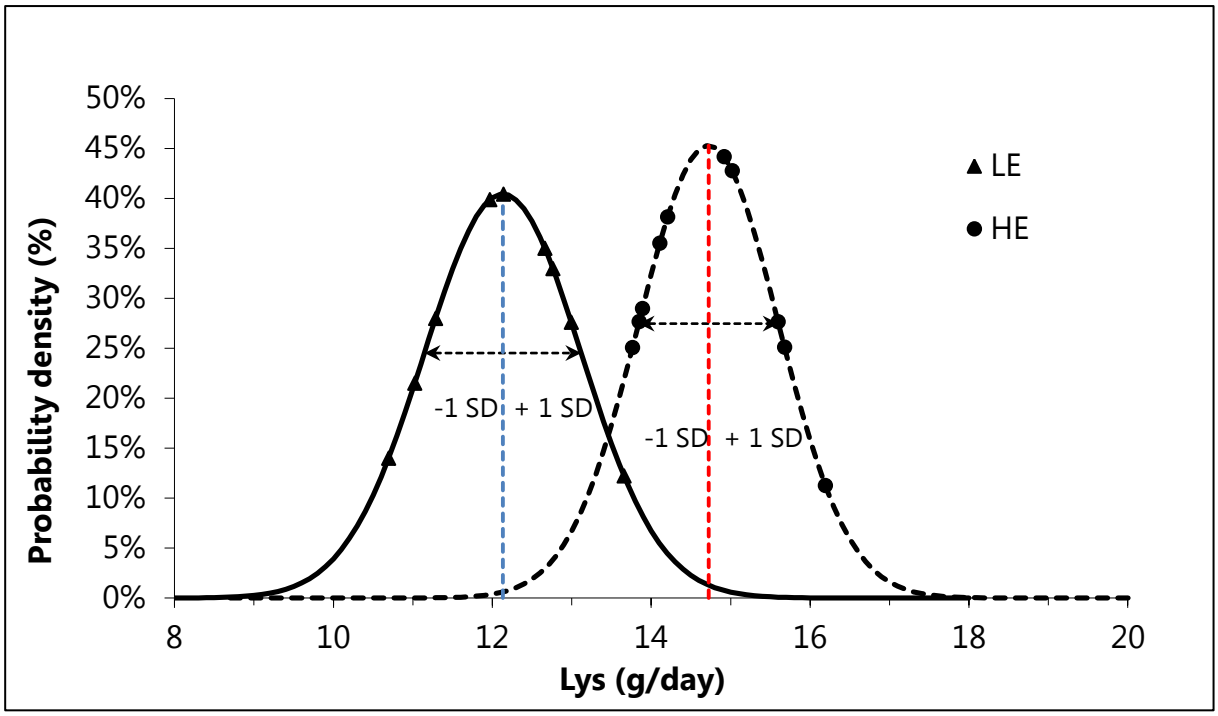


Figure 3.3 Probability density of the Lys requirement of pigs supplied with low dietary energy (LE; n = 9; mean 12.1 ± 1.0 g/day) and high dietary energy (HE; n = 10; mean 14.7 ± 0.9 g/day) in Exp. 2. Symbols represent the estimated Lys requirement of individual pigs at LE (▲) or HE (●).

Table 3.5 Effect of dietary energy supply on the linear-plateau model parameter estimates describing the relationship between total Lys (g/day) and urinary N excretion (% of N intake) in growing pigs (Exp. 2).

	Energy supply[2]		
Item[1]	**LE**	**HE**	***P*-value**
n	9	10	
Intercept	83.9 ± 1.5	84.1 ± 2.4	0.95
Slope	-4.4 ± 0.2	-4.2 ± 0.2	0.44
Breakpoint	12.1 ± 0.3	14.7 ± 0.3	< 0.001
Plateau	30.3 ± 1.1	21.7 ± 0.8	< 0.001

[1]The intercept represents the urinary N excretion at zero Lys supply. The slope represents the increment in urinary N excretion (% of N intake/(g Lys/day)). The breakpoint represents the total Lys (g/day), at which the linear phase transits into the plateau phase (*i.e.*, Lys requirement). The plateau represents the minimal urinary N excretion (% of N intake).
[2]LE = low dietary energy supply (2.2 · maintenance) from 32 to 47 kg BW; and HE = high dietary energy supply (2.7 · maintenance) from 32 to 53 kg BW.

When expressing Lys intake relative to DE intake, the linear-plateau model [3] described the relationship between Lys supply (g/MJ DE) and urinary N excretion (% of intake) better ($P < 0.10$) than the linear model [4] in all 19 pigs. The estimated Lys requirement was 0.06 g/MJ DE lower ($P = 0.01$) in HE-pigs than LE-pigs, and urinary N excretion at the plateau decreased ($P < 0.001$) 8.6 percentage points in HE-pigs (Table 3.6). The incremental efficiency of N utilization for protein deposition, as indicated by the slope, increased 14.7 percentage points ($P < 0.05$) in HE-pigs than LE-pigs (Table 3.6).

Table 3.6 Effect of dietary energy supply on the linear-plateau model parameter estimates describing the relationship between total Lys (g/MJ DE) and urinary N excretion (% of N intake) in growing pigs (Exp. 2).

	Energy supply[2]		
Parameter[1]	**LE**	**HE**	***P*-value**
n	9	10	
Intercept	77.2 ± 1.6	75.2 ± 2.5	0.53
Slope	-60.5 ± 3.1	-75.2 ± 4.4	0.02
Breakpoint	0.78 ± 0.02	0.72 ± 0.01	0.01
Plateau	30.4 ± 1.0	21.8 ± 0.8	< 0.001

[1]The intercept represents the urinary N excretion at zero Lys supply. The slope represents the increment in urinary N excretion (% of N intake/(g Lys/MJ DE)). The breakpoint represents the total Lys supply, at which the linear phase transits into the plateau phase (*i.e.*, Lys requirement). The plateau represents the minimal urinary N excretion.

[2]LE = low dietary energy supply (2.2 · maintenance) from 32 to 47 kg BW; and HE = high dietary energy supply (2.7 · maintenance) from 32 to 53 kg BW.

Discussion

The results of Exp. 1 showed that the rate of adaptation of protein metabolism to changes in AA supply is affected by the order, in which graded AA levels are tested (an increasing or a decreasing supply strategy). The HL-pigs required less time to adapt to changes in dietary Lys supply than LH-pigs. In addition, the Lys requirement estimate of LH-pigs was greater than HL-pigs. In Exp. 2, the dose response technique was proven to be accurate in estimating a quantitative change in the requirement of a limiting AA for protein deposition of individual meal-fed pigs. In both experiments, diets were formulated to be first limiting in Lys and second limiting in energy.

Methodological issues in adaptation to a different AA supply

Metabolic adaptation to changes in AA supply includes changes in the rates of protein synthesis, protein breakdown, AA oxidation, and urea production. As mentioned before, Das and Waterlow (1974) have demonstrated that enzymes involved in the urea cycle (arginase, argininosuccinate lyase, argininosuccinate synthetase, Glu

dehydrogenase, and Ala and Asp aminotransferases) adapt without lag time to a reduction in dietary protein level from 230 to 50 g/kg, whereas a period of 6 h was required to adapt to an increase in dietary protein level from 50 to 230 g/kg. This indicates that metabolic adaptation to a decreasing protein supply is more rapid than to an increasing protein supply. Pigs also adapted more rapidly to a decreasing AA supply in the current study, with responses being measured in daily rather than hourly intervals. When the balance in dietary AA is restored by supplementing Lys, protein synthesis and breakdown rates increase, resulting in an increase in whole body N turnover rate (Salter *et al.*, 1990; Rivera-Ferre *et al.*, 2006). During the Lys limiting phase in HL-pigs, the observed increase in rates of protein synthesis, breakdown, and whole body N turnover with increasing Lys supply corresponded with results of Rivera-Ferre *et al.* (2006). They found an increase in protein synthesis (+5.5 g N/kg$^{0.75}$/day), protein breakdown (+4.0 g N/kg$^{0.75}$/day), and whole body N turnover (+4.9 g N/kg$^{0.75}$/day)/(g Lys/MJ DE) when increasing the dietary Lys supply from 0.31 to 0.91 g/MJ DE in pigs with a modern genotype. In contrast, the increase in protein synthesis, breakdown, and whole body N turnover in LH-pigs in our study was 47, 40, and 33%, respectively, of those observed by Rivera-Ferre *et al.* (2006).

Considering the findings on the estimated Lys requirement, if changes in each of the adaptation processes would occur without delay, HL- and LH-pigs would have identical Lys requirement estimates. In Exp. 1, however, the Lys requirement estimate of LH-pigs was greater than HL-pigs. The lower increment in protein synthesis, breakdown, and whole N turnover rates in LH pigs and the difference in estimated Lys requirement indicate that, especially, LH-pigs require more time to metabolically adapt to changes in Lys supply than HL-pigs. The indication that LH-pigs required more time to adapt to changes in Lys supply than HL-pigs would indicate that no equilibrium in N excretion was achieved in this group. The total N excretion in urine was, however, similar between collection days and between Lys supply strategy treatments, indicating that an equilibrium was achieved within 4 d. Considering the rather slow turnover rate of the urea pool, *i.e.*, a half-life of approximately 5 h in growing pigs (Reeds *et al.*, 1987), a 4-d time period of adaptation seems sufficient. Plasma urea N reached a new equilibrium in 2 to 3 days after changing the level of dietary Lys from 0.60 or 0.90 to 0.75% or *vice versa*, thus, independent of the direction of change (Coma *et al.*, 1995). Similarly to plasma urea N, urinary urea excretion reached a new equilibrium within 3 days after adding dietary protein or AA that were limiting protein deposition (Brown and Cline, 1974; Fuller *et al.*, 1979). Although in the present study, an equilibrium in the response variables may have been achieved after changing the dietary Lys supply, the estimated requirement estimate was still different between LH and HL pigs.

This stresses the importance of taking into account the direction of change in AA supply during any dose-response study. In some N-balance studies, the supply of the limiting AA was increased over time (Heger *et al.*, 2008, 2009), whereas others have used AA titration studies applying random allocation to each AA level in time (Bertolo *et al.*, 2005; Moehn *et al.*, 2008). Although random allocation may be favored from a statistical point of view, abrupt and large changes in AA supply will probably induce

more pronounced changes in protein turnover and urea synthesis. As a consequence, a longer period of adaptation to each level of Lys supply (*i.e.*, step length) would be required to reduce the error of measurement, prolonging the dose-response study. Therefore, a decreasing Lys supply strategy was preferred over an increasing Lys supply strategy in Exp. 2.

In the current studies, the Lys supply was gradually changed in small steps (0.12 g Lys/MJ DE per step in Exp. 1 and 0.09 g Lys/MJ DE per step in Exp. 2), so that abrupt changes in Lys supply were minimized, which would likely reduce the time required to adapt to new Lys levels. To increase the precision of the Lys requirement estimate, 9 Lys supply levels were used in Exp. 2. The length of each titration step (*i.e.*, Lys level) was reduced from 4 to 3 days to reduce the length of the experimental period. As illustrated by the low CV of the Lys requirement estimates in Exp. 1 (4.9% in LH-pigs and 3.0% in HL-pigs) and 2 (8.1% in LE-pigs and 6.0% in HE-pigs), these procedures allowed us to establish high precision estimates of changes in Lys requirements in individual pigs.

The linear-plateau model [3] described the data better than the linear model [4] in 79% (Exp. 1) and 100% (Exp. 2) of the pigs, allowing estimation of Lys requirement values (*i.e.*, at the inflection point). It should be realized, however, that exclusion of pigs with a poor fit with the linear-plateau model [3] may have led to underestimation of the Lys requirement for that particular treatment. A poor fit indicates that the inflection point was not achieved within the range of Lys supply, or that the transition from a decrease in urinary N excretion to a plateau occurred at 1 of the greatest levels of Lys supply. Care should, therefore, be taken in excluding pigs from the experiment, or, preferably, in determining the range of AA levels to be tested in such titration studies. For future studies, we suggest to include at least 7, and preferably 9, AA levels, equally spaced around the expected requirement values.

In conclusion, our results demonstrated that a decreasing rather than an increasing Lys supply strategy with 7 to 9 Lys levels surrounding the expected requirement value is preferred to estimate (changes in) Lys requirements of individual meal-fed growing pigs. Similar approaches can be used for other indispensable AA.

Accuracy: effect of dietary energy supply

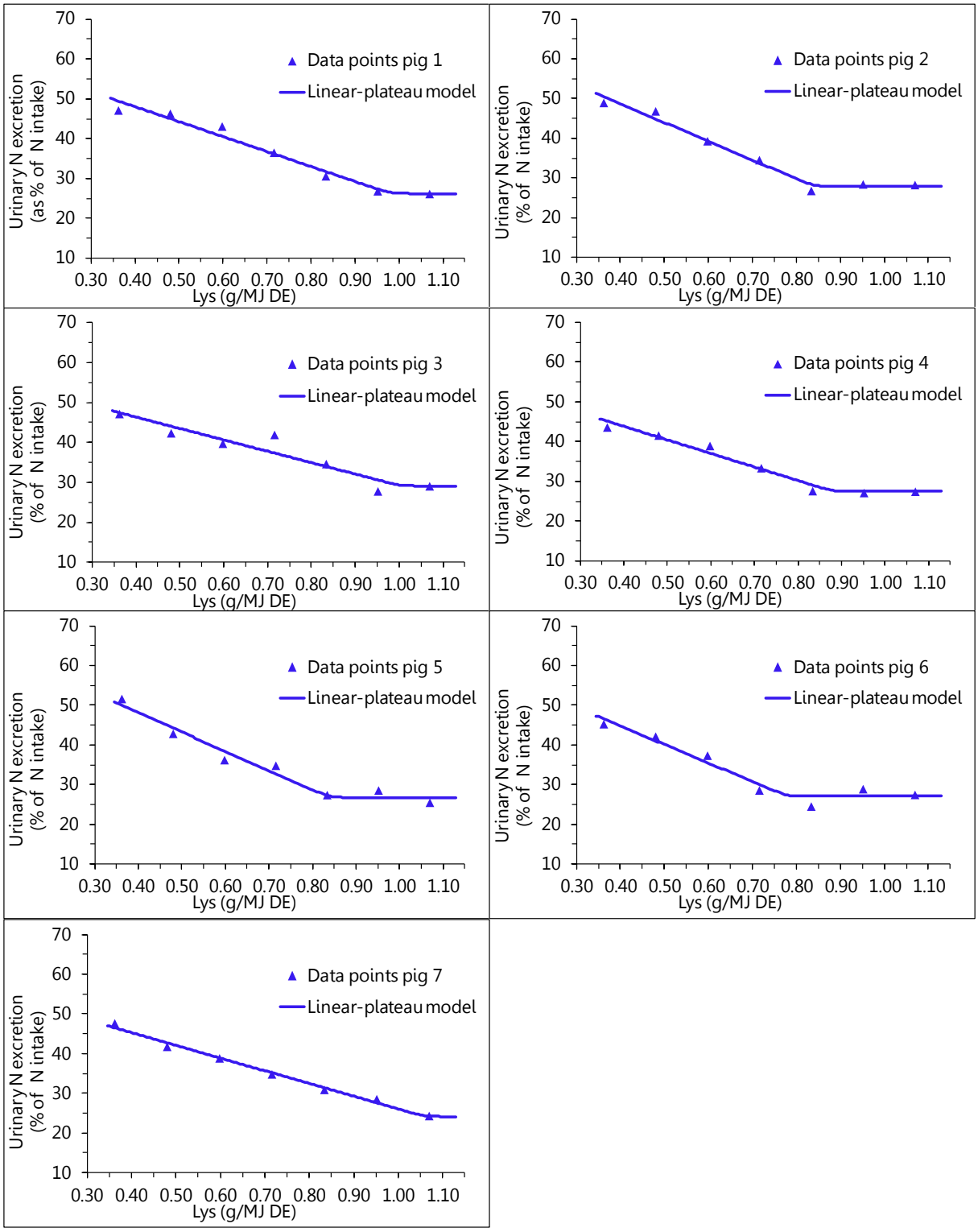
In Exp. 2, the accuracy of the dose-response technique to determine a shift in Lys requirement for individual pigs was assessed by creating a contrast in dietary energy supply. This approach has been well established to increase protein deposition, provided that the maximum rate of protein deposition has not been reached and that other indispensable AA or other nutrients are not limiting the rate of protein deposition (Campbell *et al.*, 1984; Bikker *et al.*, 1994). A previous study in growing pigs (Bikker *et al.*, 1994) reported that Lys requirements increased by 1.9 g/day when increasing the dietary energy supply from 2.5 to 3.0 · M. In addition, an increase in Lys requirement of 2.2 g/day (from 10.2 to 12.4 g/day) was predicted for this contrast in dietary energy supply by using a simulation model for growing pigs (van Milgen *et al.*,

2008). According to NRC (2012), the Lys requirement is 16.9 g/day for an ad libitum fed growing pig at an average BW of 35 kg. This results in Lys requirements of 11.8 g/day (2.2 · M) and 14.5 g/day (2.7 · M), when extrapolating from ad libitum feed intake of approximately 20.8 MJ/day to 2.2 · M (14.5 MJ/day) or 2.7 · M (17.8 MJ/day). The increase of 2.7 g/day corresponds with the shift in Lys requirement (+2.6 g/day) with greater energy supply by adding dietary starch in Exp. 2. The AA requirement values in growing pigs are influenced by many factors, including genotype, BW, health status, dietary energy supply, sex, and environment (Susenbeth, 1995; NRC, 2012). The simple AA dose-response technique can be successfully adopted to quantify a change in the AA requirements of growing pigs, as induced by these factors.

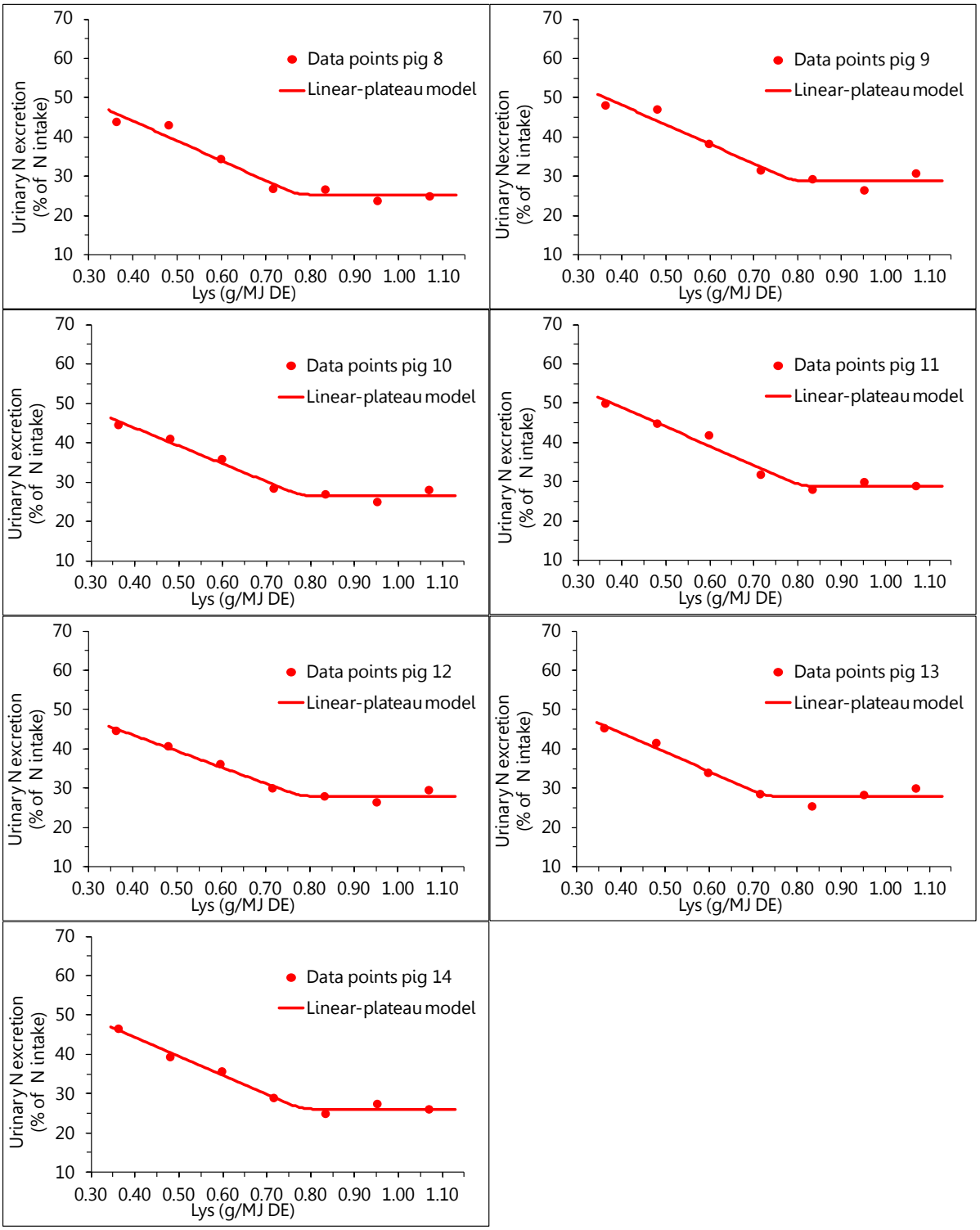
In Exp. 2, increasing the dietary energy supply by 0.5 · M decreased the Lys requirement from 0.78 to 0.72 g Lys/MJ DE, increased the incremental efficiency of Lys utilization for protein deposition from 60.5 to 70.5%, and increased the maximum efficiency from 69.6 to 78.2%. A decrease in Lys requirement (-0.03 g/MJ DE) with increasing energy supply (+ 0.5 · M) has been reported in pigs of 15 kg BW, but not in pigs of 20 and 25 kg BW (Urynek and Buraczewska, 2003). In that study, the average feeding level increased with BW (15 kg: 3.0 · M; 20 kg: 3.3 · M; and 25 kg: 3.5 · M), indicating that the reduction in Lys requirement with greater dietary energy supply may be more pronounced at lower feeding levels. In growing pigs, the ratio between lipid and protein deposition increases from 0.55 to 0.77 g/g with 0.5 · M extra energy intake (Bikker *et al.*, 1994). This may have contributed to the reduced Lys requirement with greater dietary energy supply in the present study, *i.e.*, a greater incremental deposition of lipids than of proteins. At a greater energy intake the maintenance requirements are proportionally more diluted because of a greater increase in requirements for protein deposition. As the requirement for Lys to energy ratio for protein deposition is greater than that for maintenance (NRC, 2012), an increase in the requirement for Lys to energy ratio because of the dilution effect will probably be more pronounced at greater energy intake levels. These opposing effects (increased lipid to protein deposition and "dilution" of the requirement for maintenance) could explain the lower Lys requirement value relative to energy intake at lower feeding levels in the study of Urynek and Buraczewska (2003), and the absence of an effect in the study of Bikker *et al.* (1994) at a greater feeding level than in the current study.

In conclusion, the present studies demonstrated that a dose-response technique with urinary N excretion as response variable with a low number of pigs per treatment (n = 4 to 10) provides a simple, accurate technique to estimate a quantitative change in the requirement of a limiting AA for protein deposition of individually meal-fed pigs. Potential applications of this technique include the quantification of effects of various factors, such as health status and genotype, on changes in AA requirements of pigs. Moreover, by estimating the requirement per individual pig, this technique also provides information on the variation between pigs, which could be adopted in future feeding strategies or breeding programs.

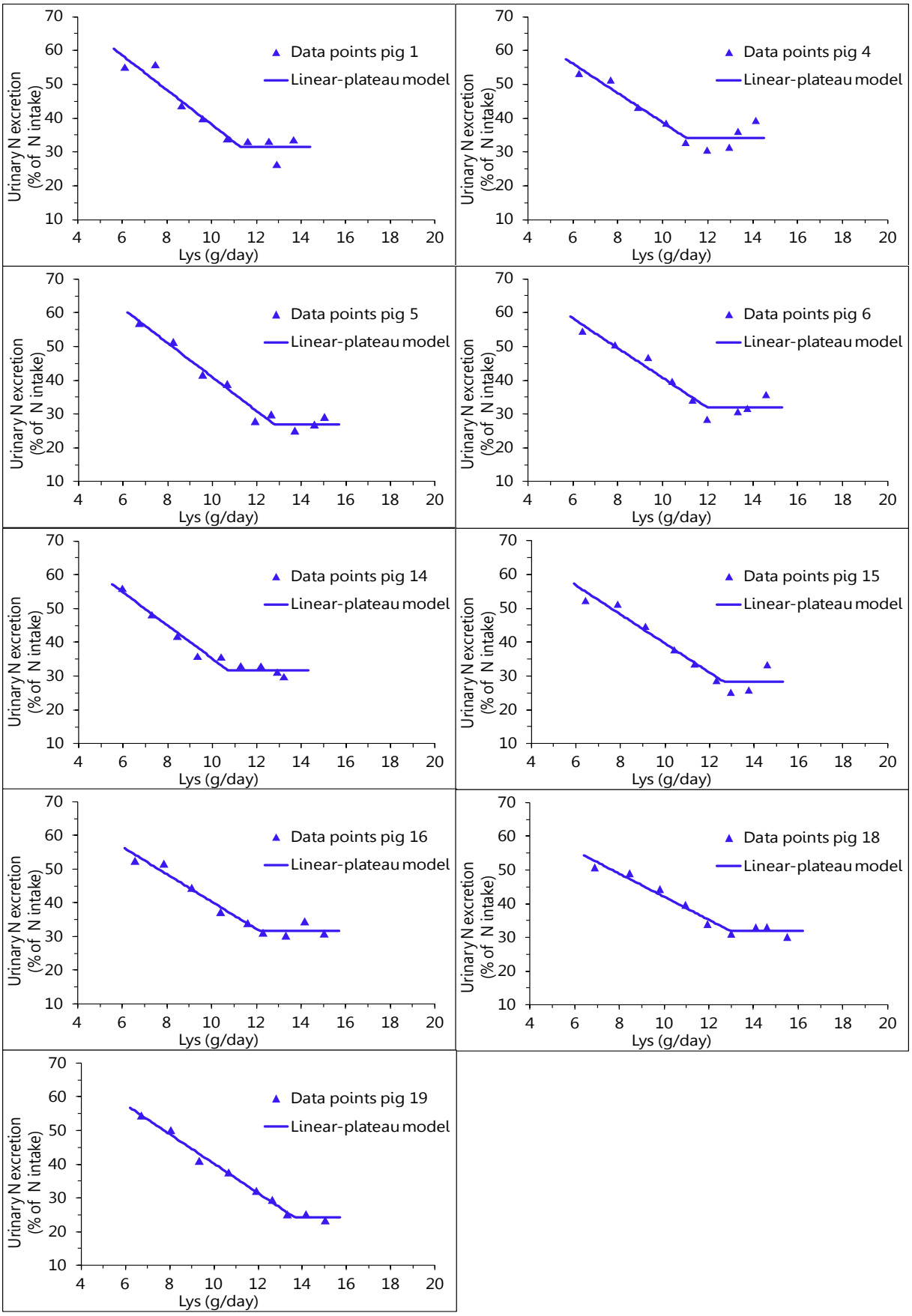
Supplemental Material



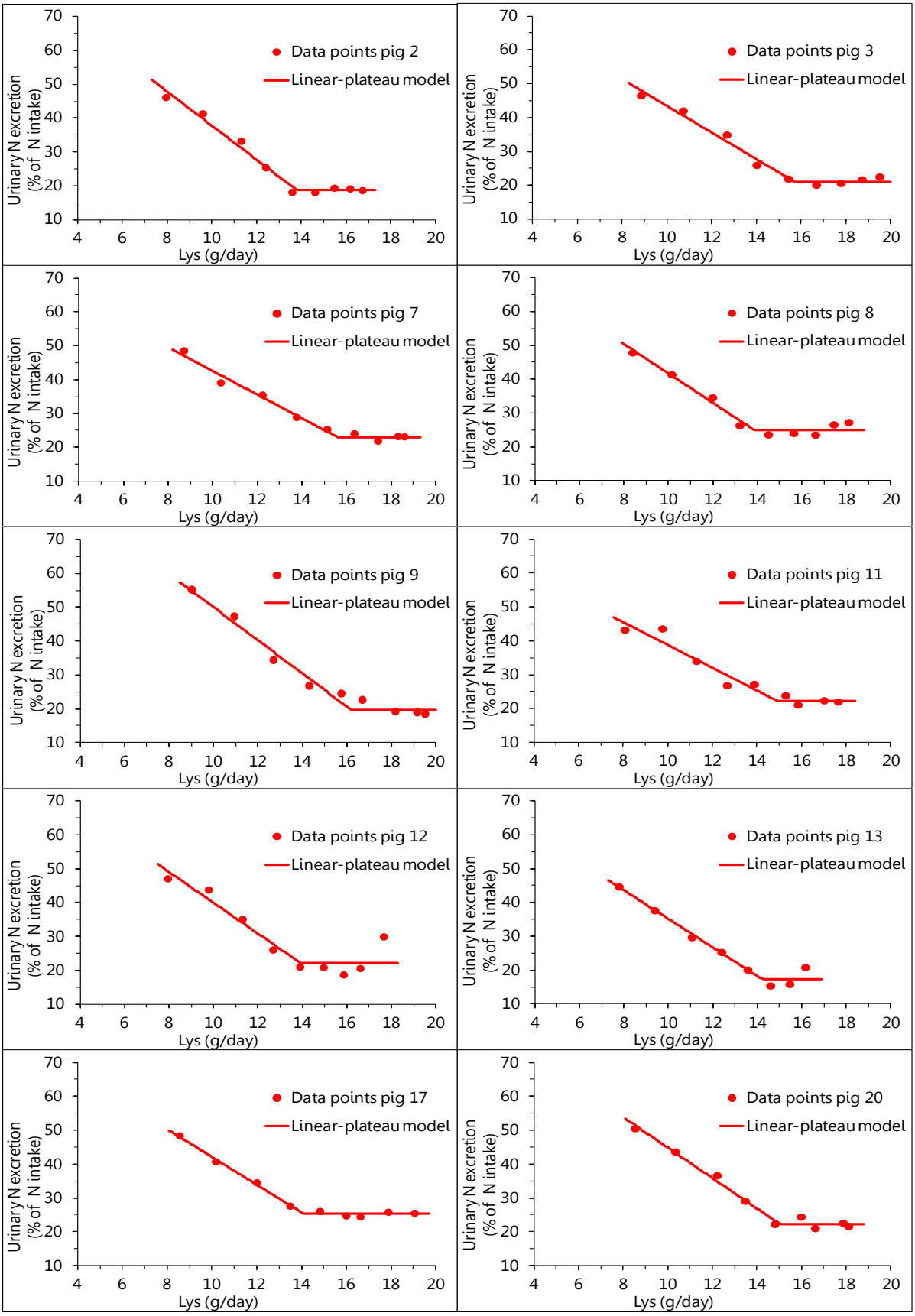
Supplemental Figure 4.1 Effect of dietary Lys supply on urinary N excretion (% of N intake) for low to high (LH)-pigs (increasing Lys supply strategy, Exp. 1). Each data point (▲) represents the mean urinary N excretion of the last 2 days at each Lys supply level period (in g/MJ DE).



Supplemental Figure 4.2 Effect of dietary Lys supply on urinary N excretion (% of N intake) for high to low (HL)-pigs (decreasing Lys supply strategy, Exp. 1). Each data point (●) represents the mean urinary N excretion of the last 2 days at each Lys supply level period (in g/MJ DE).



Supplemental Figure 4.3 Effect of dietary Lys supply on urinary N (% of N intake) for low energy (LE)-pigs (LE supply, Exp. 2). Each data point (▲) represents the mean urinary N excretion of the last 2 days at each Lys supply level period (in g/day).



Supplemental Figure 4.4 Effect of dietary Lys supply on urinary nitrogen (N) (% of N intake) for high energy (HE)-pigs (HE supply, Exp. 2). Each data point (●) represents the mean urinary N excretion of the last 2 days at each Lys supply level period (in g/day).

Erratum for Chapter 3 "A simple amino acid dose-response method to quantify amino acid requirements of individual meal-fed pigs"

Esther Kampman - van de Hoek

Incorrect statement and clarification

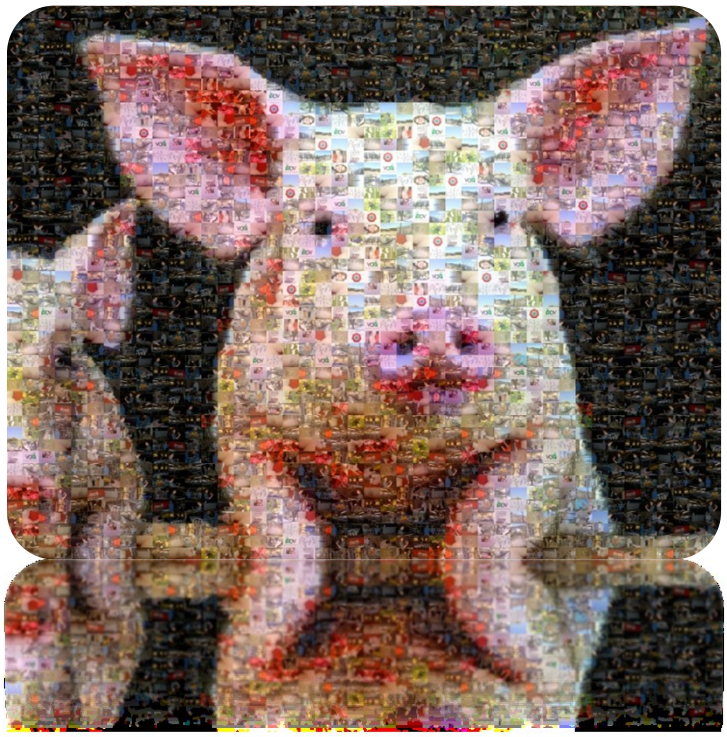
p. 67 Table 3.6 and p. 70 2nd paragraph

It is observed that increasing the dietary energy supply by 0.5 · the energy requirements for maintenance decreases the optimal Lys/DE ratio (breakpoint) from 0.78 to 0.72 g Lys/MJ DE. It is erroneously concluded that the Lys requirement per unit of DE is therefore reduced. It is possible that, at the observed breakpoint of 0.72 g Lys/MJ DE, other indispensable amino acids (e.g. threonine) have been limiting protein deposition in pigs receiving a dietary energy supply of 2.7 · the energy requirements for maintenance (High energy, HE treatment).

This erratum does not concern the conclusions of the manuscript.

Chapter 4

Dietary amino acid deficiency reduces the utilization of amino acids for growth in growing pigs following a period of low health as characterised by serum antibody presence and hygienic environment



Esther Kampman - van de Hoek[a,b], Alfons J.M. Jansman[a], Joost J.G.C. van den Borne[b], Carola M.C. van der Peet-Schwering[a], Hetty van Beers-Schreurs[c], and Walter J.J. Gerrits[b]

Submitted to Journal of Nutrition

[a]WUR Livestock Research, P.O. Box 338, 6700 AH Wageningen, The Netherlands
[b]Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands
[c]SDa, Autoriteit Diergeneesmiddelen, Yalelaan 114, 3584 CM Utrecht, The Netherlands

Abstract

During immune system activation, partitioning of amino acids (AA) between protein gain and immune system functioning changes. Our aim was to determine the effects of health status and dietary AA deficiency on nitrogen (N) retention and AA utilization in growing pigs. Castrated pigs (55 ± 0.4 days of age) were obtained from a farm of high health (HHS, n = 14) or low health status (LHS, n = 14), allocated to a diet adequate in essential AA (Adq) or deficient in Met + Cys, Thr and Trp (Def). Upon arrival, LHS pigs had greater haptoglobin, lower albumin and greater leukocyte counts ($P < 0.01$) than HHS pigs (all $P < 0.01$), but LHS pigs showed signs of recovery during the trial. Total tract N digestibility was lower in LHS pigs (4%, $P < 0.01$). LHS-Adq pigs, showed compensatory body weight gain upon arrival, coinciding with a greater N retention ($P < 0.01$) and greater efficiency of N utilization ($P < 0.001$) in LHS than HHS pigs. LHS pigs had increased ILR for Lys. Health status · diet interactions for Lys ($P = 0.07$), Val ($P = 0.03$), Leu ($P = 0.10$) pool size, and an increased urea pool size in LHS pigs ($P = 0.01$) support the observation that the increase in ILR of Lys in LHS pigs relates to oxidation when feeding the AA-Def, but to synthesis when fed the AA-Adq diet. This study illustrates how the competition for AA between synthesis of proteins associated with immune system activation and body protein deposition is enlarged when dietary supply of Met + Cys, Thr and Trp is limiting in pigs during and following a period of low health.

Keywords: health status; dietary amino acid deficiency; irreversible loss rate; urea entry rate; nitrogen retention; growing pigs.

Introduction

During immune system activation, an increased competition occurs for amino acids (AA) between body protein deposition and immune system functioning (Klasing and Johnstone, 1991; Sandberg *et al.*, 2007). The concentration of serum acute phase proteins (APP) in pigs, for example, can increase more than tenfold in response to infection or experimentally induced inflammation (Petersen, 2004; Heegaard *et al.*, 2011). The AA composition of APP, however, differs largely from the composition of muscle protein (Reeds *et al.*, 1994). As a consequence, the increased demand for especially Phe, Trp and Tyr for the synthesis of APP, may lead to an imbalance in AA available for body protein deposition, resulting in increased AA oxidation and N loss in urine (Reeds *et al.*, 1994). The estimated N loss derived from the oxidation of unbalanced AA due to excessive demands for aromatic acids for the synthesis of APP is close to the N loss observed under conditions of infection and trauma (Reeds *et al.*, 1994). Moreover, the cytokine induced metabolic change after immune system activation generally results in increased breakdown and decreased synthesis of skeletal muscle protein (Zamir *et al.*, 1992; Breuille, 1999). Several studies have shown that immune system activation reduces feed intake, daily weight gain, feed efficiency, and protein deposition in pigs (Williams *et al.*, 1997; Le Floc'h *et al.*, 2006; Pastorelli *et al.*, 2011). Immune system activation reduced plasma Trp concentrations (Melchior *et al.*, 2004; Le Floc'h *et al.*, 2010) and the efficiency of Trp utilization for body protein deposition in pigs (de Ridder *et al.*, 2012). The absolute requirement, *i.e.* in g/day, for Lys (Williams *et al.*, 1997), Met + Cys decreased after immune system activation (Rakhshandeh *et al.*, 2014). Traditional dose-response studies typically estimate the requirement of a single AA, but do not provide insight in simultaneous changes in the utilization of other AA. Measuring the plasma irreversible loss rate (ILR, *i.e.* the amount of free AA that disappears per unit of time from the plasma pool for protein synthesis or oxidation) can be performed for multiple AA simultaneously, allowing estimation of a shift in metabolism of different AA. We hypothesize that a reduced health status leads to a shift in AA utilization, due to changes in the partitioning of AA towards synthesis of proteins of the immune system at the expense of synthesis of proteins in muscle. In addition, there is increasing evidence that a deficient dietary AA supply can impair cell-mediated responses of the immune system, thereby reducing the resistance to pathogens and the ability to regulate the immune system in response to disturbances (Grimble, 2001; Li *et al.*, 2007; Calder and Yaqoob, 2012). The aim of the present study was to determine the effects of health status and dietary deficiency of Met + Cys, Thr and Trp on whole body N retention and AA utilization in growing pigs. We hypothesized that a dietary deficiency of Met + Cys, Thr and Trp would increase the competition for AA, hence reducing immune system functioning and body protein deposition. Combined measurements of whole body N retention and rates of irreversible loss of AA from plasma, urea entry and plasma protein synthesis (after a bolus of ^{13}C labelled AA and $^{15}N_2$ urea) were expected to provide insight into the consequences of immune system activation for AA metabolism.

Material and methods

Animals and treatments

The study was approved by the Animal Experimental Committee of Wageningen University. In total 28 barrows (Dutch Landrace · York) of similar age at arrival (55 ± 0.4 d) were used. Barrows originated from either a farm of high health (HHS; n = 14) or from a farm of low health status (LHS) (n = 14) as assessed by a serological monitoring program for the presence of antibodies against a number of pathogens in pigs (Supplemental Table 4.1). On both farms, one barrow per litter was selected from 14 litters, with a body wt (BW) around the average BW of each litter. All barrows were born in the same week. After arrival at the experimental facilities, HHS pigs were kept in metabolism cages (1.90 · 0.65 m) in disinfected respiration chambers (air temperature 23°C) with High-Efficiency Air-filters (1D-H13, Camfill KG, Reinfeld, Germany) to reduce exposure to pathogens from the environment. In addition, a strict hygiene protocol was used (including showering, changing of clothes, disinfection of hands and boots, use of a hair nets, face masks and gloves). HHS pigs received antibiotics in their diet (8.4 mg amoxicillin/kg BW/day) during 4 days after arrival. LHS pigs were kept in metabolism cages (2.10 · 0.65 m) in a cleaned but not disinfected room (air temperature 22 to 25°C) in which other pigs were housed prior to the current study. LHS pigs did not receive dietary antibiotics after arrival. A timeline of the experiment is shown in Figure 4.1 with day 0 being the start of the dietary treatment allocation. For practical reasons HHS and LHS groups were split into two batches of 7 pigs each, with a time lag of 1 day between both batches. Within each health status, pigs were allocated to a diet adequate in essential AA (Adq) or a diet deficient in Met + Cys, Thr and Trp (Def) at the start of the experiment (day 0). This resulted in four treatment groups (n = 7 each), *i.e.* HHS-Adq, HHS-Def, LHS-Adq and LHS-Def. Dietary treatments were balanced over batches of pigs. At day -1 or 0, pigs were surgically fitted with a jugular vein catheter for blood collection and a carotid artery catheter for injection of a mixture of U-^{13}C labelled AA and $^{15}N_2$ urea. Neopen (5 mg of Neomycine and 10,000 IE Procaïne benzylpenicilline per kg of BW; Intervet, Boxmeer, The Netherlands) was given *i.m.* at 1 day before surgery, at surgery and at 1 day after surgery. Flunixine (2.2 mg/kg BW, Fynadine; Schering-Plough, Brussels, Belgium) was given *i.m.* at surgery and for 2 days after surgery. Pigs were weighed at arrival, at day -1 or 0, at day 2 (denoted as 'initial'), and at day 9 (denoted as 'final').

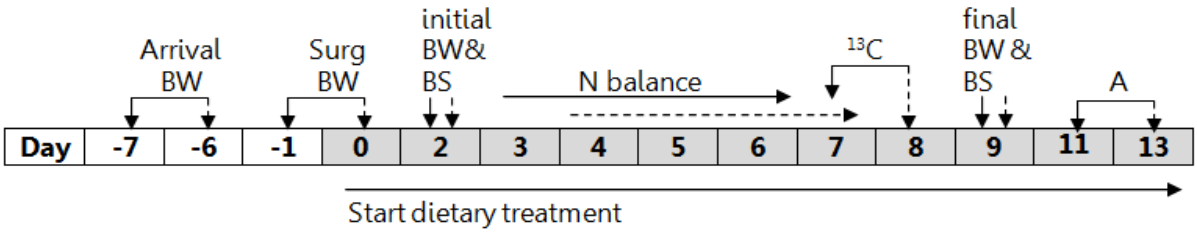


Figure 4.1 Timeline of the experimental period. A = autopsy; BS = Blood sampling; BW = BW measurement; ^{13}C = Injection of U-^{13}C labeled AA and $^{15}N_2$ urea mixture; Surg = Surgery for catheterization; ⟶ = batch 1; ⇢ = batch 2.

Experimental diets

The experimental diets are presented in Table 4.1. In order to prevent that dietary energy supply would limit protein deposition, assuming distinct protein and energy dependent phases of protein deposition in pigs (Campbell *et al.*, 1984; Bikker *et al.*, 1994), the adequate diet was designed to be first-limiting in Lys, being 95% of the requirement value for Lys. The requirements for other essential AA were met for growing pigs in the range of 25 to 45 kg BW (CVB, 2004, 2008). The deficient diet was also formulated to be marginally deficient in Lys, *i.e.* at 95% of the Lys requirement value (CVB, 2004, 2008), and was deficient in Met + Cys, Thr and Trp (all at 76% of their requirement according to CVB (2008). All pigs received the adequate diet until day 0. From day 0 until the end of the experiment HHS-Adq and LHS-Adq pigs were fed the adequate diet, and HHS-Def and LHS-Def pigs were fed the deficient diet. Diets were provided as mash and mixed with water in a ratio of 1 to 3. Pigs were fed at 2.7 · the energy requirements for maintenance (M) (458 kJ metabolizable energy/(kg BW0.75/day) according to ARC (1981). Feed allowance was adjusted for BW and BW gain was assumed to be 250 g/day. Pigs were fed their daily allowance in two equal meals, one provided at 0730 and one at 1630 h. Diets were analyzed for N content by the Kjeldahl method. Crude protein was calculated as N · 6.25 (ISO, 2005a). Diets were analyzed for AA by HPLC after hydrolysis in hydrochloric acid (ISO, 2005b,c).

Table 4.1 Composition of the experimental diets (as-fed basis).

	Adequate diet		Deficient diet
Ingredient composition		*g/kg*	
Barley	343.5		343.5
Maize	150.0		150.0
Wheat starch	146.9		150.0
Soybean meal	135.8		135.8
Wheat	97.5		97.5
Pea	75.0		75.0
Calcium carbonate	14.3		14.3
Monocalcium phosphate	8.8		8.8
Wheat gluten feed	8.0		8.0
Soybean oil	5.0		4.9
Sodium bicarbonate	2.4		2.4
Sodium chloride	2.0		2.0
Vitamin and mineral premix[1]	2.0		2.0
Potassium carbonate	1.5		1.5
L-Lys HCl	3.6		3.6
DL-Met	1.5		0.2
L-Thr	1.3		0.0
L-Trp	0.4		0.0
L-Val	0.5		0.5
Calculated nutrient composition		*g/kg*[2]	
Dry matter	873.5		873.1
NE[3], MJ/kg	9.67		9.67
Crude protein	152.4		150.4
Crude ash	50.7		50.7
Crude fat	28.4		28.3
Crude fiber	31.8		31.8
AID[4] Lys	8.7		8.7
AID Met	3.4		2.1
AID Met + Cys	5.4		4.1
AID Thr	5.2		4.0
AID Trp	1.7		1.3
AID Ile	4.9		4.9
AID His	3.1		3.1
AID Phe	6.2		6.2
AID Tyr	4.2		4.2
AID Leu	9.4		9.4
AID Val	6.0		6.0
Analyzed nutrient composition		*g/kg*	
Crude protein	154.0		154.0
Lys	10.3		10.4
Met	3.8		2.7
Cys	2.4		2.5
Trp	2.1		1.8
Thr	6.6		5.4

[1]Premix contained (per kg premix): 4,000,000 IU of vitamin A; 1,000,000 IU of cholecalciferol; 10,000 IU of vitamin E; 750.5 mg of vitamin K; 166,667 IU of thiamin; 800,000 IU of riboflavin; 6,000 mg of pantothenic acid; 10,000 mg of niacin; 10,000 μg of vitamin B-12; 100 mg of folate; 500 mg of vitamin B-6; 50,000 mg of choline chloride; 50,000 mg of Fe:FeSO4; 5,000 mg of

$Cu:CuSO_4 \cdot 5H_2O$; 32,500 mg of Zn:ZnO; 15,001 mg of Mn:MnO; 75 mg of Co:CoC; 375 mg of KI; and 150 mg of Se:Na-selenite.
[2]Unless indicated otherwise.
[3]Net energy, NE. Calculated based on CVB (2004).
[4]Apparent ileal digestible, AID.

Health status

Blood samples were taken from the jugular vein catheter to characterize the health status of the pigs. Initial (day 2) and final (day 9) blood samples were collected into EDTA tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria) and were analyzed for the number of total leukocytes and differential leukocytes by an automated counter (Cell-Dyn 3700, Abbott, Hoofddorp, The Netherlands). Initial blood samples were also collected into serum tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria) and were allowed to clot for 1 h at room temperature. Serum was collected after centrifugation for 10 min at 1800 *g* and was stored at -20°C pending analysis of albumin (Randox Bromocresol Green assay, cat. no. AB 362), haptoglobin (Tridelta Phase Haptoglobin Assay, cat. no. TP-801), pig major acute phase protein (Pig-MAP, ELISA, Reactivlab Limited, Glasgow, Scotland) and C-reactive protein (CRP, ELISA, Reactivlab Limited, Glasgow, Scotland). All LHS pigs (at day 11) and three HHS pigs (at day 13) were euthanized after which autopsy was performed, the remaining HHS pigs were used in a subsequent study. Autopsy observations included judgment of body condition, and visual inspection for abnormalities of lung, spleen, stomach, small intestine, large intestine, kidney, liver, hart, and mesenteric lymph nodes by an experienced pathologist.

Nitrogen balance

Pigs were equipped with a Velcro support system to allow separate collection of urine and feces (Van Kleef *et al.*, 1994). Feces and urine were collected quantitatively over 4 subsequent days. Feces were stored at -20°C pending analysis. Urine was collected via funnels, which were sprayed with an acetic acid buffer to prevent evaporation of NH_3, into buckets containing sulfuric acid (9N) for conservation. Urine was collected from the buckets, weighed, sampled and stored at -20°C pending analysis. Nitrogen in urine and feces was analyzed using the Kjeldahl method (ISO, 2005a). Dry matter content of feces was determined by drying at 103°C (ISO, 1999).

Amino acid metabolism

On day 7 or 8, the utilization of AA was studied by measuring the change in plasma isotopic enrichment of individual AA in time after an intravenous (*i.v.*) bolus of U-^{13}C labelled AA. This allowed calculation of the ILR of AA from the plasma. Irreversible losses of AA occur by incorporation of AA into protein (protein synthesis) or by oxidation of AA (Reeds *et al.*, 1980). To create a steady state in dietary AA supply in time during the injection of the U-^{13}C labelled AA and $^{15}N_2$ urea mixture, the daily feed

allowance on day 7 or 8 was divided over ten equal meals. Two meals were fed at 0730 h, followed by hourly meals from 0910 h until 1610 h. At 1230 h, a mixture of 11 U-^{13}C labelled AA (97 to 99 atom percent, Cambridge Isotope Laboratories, Andover, USA) and $^{15}N_2$ urea (98 atom percent, Sigma-Aldrich, St. Louis, USA) was injected. The composition of the mixture (mg/g saline) was L-Cys, 0.04; L-His, 0.08; L-Ile, 0.16; L-Leu, 0.16; L-Lys, 0.17; L-Met, 0.06; L-Phe, 0.13; L-Thr, 0.18; L-Trp, 0.07; L-Tyr, 0.16; L-Val, 0.19; and $^{15}N_2$ urea, 2.0. The mixture was injected as a bolus (0.50 g/kg BW; 0.25 mL/s) in the carotid artery. If the carotid artery was blocked, the mixture was injected in the jugular vein. Blood samples (4 mL) were collected from the jugular vein and transferred into tubes containing lithium heparin (Vacuette, Greiner Bio-One, Kremsmünster, Austria) at 20 and 10 min before injection of the isotope mixture and at 3, 6, 9, 12, 15, 20, 25, 30, 40, 50, 61, 76, 91, 120 and 181 min after injection. Tubes were immediately placed on ice and centrifuged for 10 min at 2000 *g* at 4°C, after which plasma was collected and stored at -20°C pending analysis. Nine blood samples (-10, 3, 6, 9, 12, 15, 20, 40 and 61 min after injection) per pig were used to measure ^{13}C enrichment in plasma Ile, Leu, Lys, Phe, Trp, Tyr, and Val as described by Kampman - van de Hoek *et al.* (29). The ^{13}C enrichment of Met and His could not be successfully analyzed due to high losses during the derivatization step. The ^{13C} enrichment in Thr and Cys could not be determined with the current procedure. Eight blood samples (-10, 3, 9, 20, 61, 120, 181 and 1260 min after injection) per pig were used to measure ^{15}N enrichment of plasma urea and ^{13}C enrichment in plasma proteins. For urea enrichment, the plasma samples were deproteinized by mixing 0.3 mL sodium tungstate (10% w/w) and 0.2 mL sulphuric acid (1N) with 400 µL plasma. After centrifugation, the supernatant was transferred into a 2-mL tube and the precipitate was used for measuring ^{13}C enrichment in plasma protein. 400 µL Dowex ion exchange resin (Ag 50W-X8 H+ form, 200-400 mesh, Dow Chemical Company, Edegem, Belgium) was added to the supernatant. After centrifugation the precipitate was flushed with Millipore water and collected. After evaporation to dryness, the sample was evaporated with a centrifugal concentrator (Jouan RC 1022, Thermo Scientific, Waltham, USA). The precipitate was used for measuring ^{13}C enrichment in plasma proteins after freeze-drying. ^{15}N enrichment in plasma urea and ^{13}C enrichment in plasma protein was measured after combustion in an elemental analyzer (Flash 2000 organic elemental analyzer HT O/H-N/C, Thermo Scientific, Bremen, Germany) using a continuous flow isotope ratio mass spectrometer (Conflo IV, Thermo Scientific, Bremen, Germany).

Models

Assuming a physiological steady state conditions during the measurements, ILR and urea entry rate were calculated from the change in respectively plasma AA and urea isotopic enrichment after an *i.v.* bolus of U-^{13}C labelled AA and $^{15}N_2$ urea using the model and calculations as described by Holtrop *et al.* (2004). Furthermore, it is assumed that there is no recycling of tracer into the plasma pool and that the tracer transfers along with the tracee between compartments with a constant fractional rate (Waterlow, 2006); the ILR of an AA occurs as an output from the plasma pool, and only

by incorporation of the AA into protein or by oxidation (Reeds *et al.*, 1980). For each AA and pig, a double exponential model was fitted to the ^{13}C enrichment in plasma AA after administration of the bolus injection:

$$E(t) = a1 \cdot \exp(b1 \cdot t) + a2 \cdot \exp(b2 \cdot t) \quad [1]$$

where E(t) is the predicted ^{13}C enrichment in plasma AA (TTR) at time t (min), and a1, b1, a2, and b2 are parameter estimates. The double exponential model [1] was also fitted to the ^{15}N enrichment in plasma urea under the assumption that a two compartment model is more appropriate than a single model as indicated in growing pigs (Oosterveld *et al.*, 2005) and humans (Matthews and Downey, 1984). For ^{13}C enrichment of the plasma protein fraction (TTR, corrected for background enrichment), for each pig, a linear model without intercept was fitted to the measured ^{13}C enrichment in plasma protein in time after administration of the bolus injection. The slope reflects the rate of plasma protein synthesis from free plasma AA during the measurement period.

Calculations

Average daily gain (ADG), dietary N intake, faecal and urinary N excretion and N retention were expressed relative to BW to correct for differences in BW between treatment groups (BW at arrival was 19.5 ± 0.32 for HHS and 13.6 ± 0.50 kg for LHS pigs). ^{13}C enrichment in plasma AA and protein, and ^{15}N enrichment in plasma urea was expressed as tracer-to-tracee ratio (TTR), and background enrichment (obtained from plasma samples taken before injection of the mixture of labelled AA and urea) was subtracted. Pig was considered as the experimental unit. With the parameter estimates derived from the exponential model [1] the ILR (µmol/(kg BW·h)) [2] and pool sizes [3] were calculated:

$$ILR = do / (a1 / b1 + a2 / b2) \cdot 60 \quad [2]$$

$$\text{Pool size} = do / (a1 + a2) \quad [3]$$

where do is the dose of the U-^{13}C labelled AA (µmol/kg BW).

AA released from protein breakdown was calculated as the difference between ILR and intake, using the steady state model (Waterlow *et al.*, 2006), *i.e.* ILR = protein breakdown + dietary intake = protein synthesis + AA oxidation. Urea entry rate and pool size were calculated with formula [2] and [3] respectively, with do being the dose of the ^{15}N labelled urea (µmol/kg BW).

Statistical analyses

The goodness of fit of the double exponential model was assessed by computing the mean square prediction error (MSPE). The root MSPE was scaled to the observed mean (mean prediction error) and the correlation between predicted and observed values was calculated. Errors due to overall bias, errors due to deviation of the regression slope from unity, and errors due to random variation were calculated (Bibby and

Toutenburg, 1977). All variables and parameter estimates were analyzed by ANOVA with health status and dietary treatment as fixed effects. To test whether the change in ^{13}C enrichment (TTR) of plasma protein as reflected by the slope was different from zero, *i.e.* whether or not the ^{13}C enrichment (TTR) of plasma protein increased in time, the relation between TTR and time was analyzed by ANOVA. In addition, effect of health status, dietary treatment and day of collection (initial or final) on the count of total leukocytes, neutrophils, lymphocytes, monocytes and the sum of eosinophils and basophils were analyzed with a mixed model with collection day within pig taken as repeated measures. Apart from monocyte counts, dietary treatment did not affect the leukocyte counts. Therefore the presented data do not include the effect of dietary treatment. A covariance structure was chosen based on the lowest value for the Akaike and Bayesian information criteria. The normality of the distribution of studentized residuals was assessed. Data on the granulocyte count were log transformed to obtain normal distribution of model residuals. All statistical procedures were conducted in SAS (SAS Inst. Inc., Cary, NC). Values are presented as means ± (pooled) SEM, and effects were considered significant at $P \leq 0.05$.

Results

Data of one pig in the HHS-Def treatment were excluded due to feed refusals, occurrence of fever and lung and liver abnormalities observed in a subsequent study, explaining an extremely low ADG and N retention in the present experiment. Data of two pigs in the LHS-Def treatment were excluded due to an error in BW determination and therefore incorrect feed allowances.

Health status

At the initial blood collection (day 2), LHS pigs had a lower ($P < 0.001$) serum albumin concentration than HHS pigs and a greater ($P < 0.001$) serum haptoglobin concentration, resulting in a greater ($P < 0.001$) haptoglobin to albumin ratio (Table 4.2). Serum total protein concentration tended to be greater ($P = 0.07$) in LHS pigs than in HHS pigs (Table 4.2). Serum Pig-MAP and CRP concentrations did not differ between LHS and HHS pigs. Dietary treatment did not affect serum APP concentrations, although the serum haptoglobin concentration ($P = 0.09$) and the haptoglobin-to-albumin ratio ($P = 0.07$) tended to be greater in LHS-Def than in LHS-Adq pigs, but not in HHS pigs (Table 4.2).

LHS pigs had greater counts of total leukocytes ($P < 0.001$), granulocytes (sum of neutrophils, eosinophils and basophils) ($P < 0.001$) and lymphocytes ($P < 0.01$) than HHS pigs (Table 4.3). Counts of total leukocytes ($P < 0.05$), and granulocytes ($P < 0.01$) were lower at the final than at initial blood collection (Table 4.3). Dietary treatment did not affect counts for total leukocytes, granulocytes and lymphocytes, but monocyte count was greater ($P = 0.01$) in Def pigs ($0.91 \pm 0.081 \cdot 10^9$/L) than in Adq pigs ($0.68 \pm 0.054 \cdot 10^9$/L). The effect of dietary AA supply on monocyte count was numerically

more pronounced in LHS pigs (52% greater in LHS-Def than in LHS-Adq) than in HHS pigs (18 % greater in HHS-Def than in HHS-Adq).

Autopsy revealed that 13 out of 14 LHS pigs showed abnormalities, including 1 pig with signs of local necrotic pneumonia, 4 with signs of pneumonia by *Actinobacillus pleuropneumoniae*, 1 with signs of chronic pleuritis, 4 with signs of pneumonia, 1 with signs of *Escherichia coli* diarrhea, 1 with signs of kidney inflammation, and 1 with signs of a *Staphylococcus aureus* infection, whereas no abnormalities where found in HHS pigs (n = 3).

Table 4.2 Effect of health status and dietary AA supply on serum acute phase proteins and serum total protein concentrations at day 2 in growing pigs.

Health status (H)	**High Health (HHS)**		**Low Health (LHS)**			***P*-value**		
Dietary AA supply (D)	**Adq n = 7**	**Def n = 6**	**Adq n = 7**	**Def n = 5**	***SEM***	**H**	**D**	**H · D**
Albumin, g/L	35.2	34.2	30.5	30.5	*0.61*	< 0.001	0.59	0.56
Haptoglobin, g/L	1.3	1.1	2.0	2.6	*0.15*	< 0.001	0.43	0.09
Hapt. / Alb.·100	3.6	3.1	6.7	8.4	*0.50*	< 0.001	0.27	0.07
Pig-MAP, g/L	1.3	1.5	1.3	1.5	*0.08*	0.83	0.30	0.86
CRP, mg/L	363	383	442	339	*23.6*	0.72	0.40	0.21
Total protein, g/L	48.6	49.3	51.4	51.6	*0.68*	0.07	0.73	0.83

Table 4.3 Effect of health status and day of collection on blood leukocyte counts in growing pigs at day 2 (initial collection) and day 9 (final collection).

Health status (H)	**HHS**		**LHS**			***P*-value**		
Day	**Initial**	**Final**	**Initial**	**Final**	***SEM***	**H**	**Day**	**H · Day**
n	13	12	12	12				
Leukocytes, 10^9/L	17.2	17.0	26.0	22.5	*0.77*	< 0.001	0.03	0.09
Granulocytes[1], 10^9/L	8.4	7.4	14.4	11.3	*0.65*	< 0.001	< 0.01	0.57
Lymphocytes, 10^9/L	8.0	8.8	11.0	10.3	*0.35*	< 0.01	0.90	0.21
Monocytes, 10^9/L	0.8	0.8	0.7	0.9	*0.05*	0.96	0.26	0.14

[1]Granulocytes are the sum of neutrophils, eosinophils and basophils; log-transformed to obtain normal distribution of residuals.

Performance, Nitrogen balance and digestibility

Results of performance, N balance and total tract digestibility are shown in Table 4.4. LHS pigs had greater (P < 0.01) faecal N excretion, and a lower (P < 0.01) urinary N excretion than HHS pigs. Coinciding, LHS pigs had greater (P < 0.01) N retention than HHS pigs. The dry matter (DM) content of the feces was lower (P < 0.01) in LHS pigs than in HHS pigs and lower (P = 0.05) in Def than Adq pigs. Apparent total tract digestibility of DM (P < 0.05) and N (P < 0.01) was lower in LHS pigs than in HHS pigs.

The utilization of digestible N for protein deposition was greater ($P < 0.001$) in LHS than in HHS pigs. Initial BW (at day 2) and final BW (at day 9) were lower ($P < 0.001$) in LHS pigs than in HHS pigs. ADG (in g/day and expressed relative to BW) and gain-to-feed ratio were greater ($P < 0.01$) in LHS-Adq than LHS-Def pigs, but similar in HHS-Adq and HHS-Def pigs. ADG was 112 g/day higher ($P < 0.01$) in LHS-Adq than in LHS-Def pigs, but this effect of diet was absent in HHS pigs (H · D, $P < 0.01$). When expressed relative to BW, ADG in LHS-Adq pigs also exceeded ADG in HHS pigs ($P = 0.02$), and ADG in LHS-Def pigs exceeded ADG in HHS-Adq pigs ($P = 0.02$), but not HHS-Def pigs.

Protein metabolism

The double exponential model accurately described the decrease in ^{13}C enrichment of individual plasma AA after injection of the ^{13}C AA bolus. An example of a curve fit is presented in Supplemental Figure 4.1. The average root MSPE of the seven studied AA ranged between 2.8 and 3.9%, with > 99% of the prediction error attributable to random variation. ILR was greater ($P < 0.05$) for Lys and tended to be greater for Ile ($P = 0.08$) in LHS than in HHS pigs (Table 4.5). ILR for Trp tended to be greater ($P = 0.06$) in Adq pigs than in Def pigs (Table 4.5). Lys pool size tended to be greater ($P = 0.07$) in LHS-Def than in HHS-Def pigs. Val pool size was lower in HHS-Def than in HHS-Adq, but greater in LHS-Def than in LHS-Adq pigs (H · D; $P = 0.03$). Tyr pool size tended to be lower ($P = 0.07$) in Def pigs than Adq pigs. Urea pool size was greater ($P = 0.01$) in LHS pigs than in HHS pigs. Urea entry rate was not affected by health status or dietary treatment.

For ^{13}C enrichment in plasma protein, a linear model was fitted to from 20 min until 120 min after isotope injection. Data of the first 20 min after isotope injection were discarded to prevent contamination of samples with traces of ^{13}C AA from the catheter lines and to allow homogenous distribution of the tracer in the plasma AA pool (*i.e.* unrealistically high TTR in first two samples after injection). Incorporation of label into the plasma protein pool was detected, but the slope of ^{13}C enrichment in plasma protein in time was not affected by health status or dietary treatment (Table 4.5).

Table 4.4 Effect of health status and dietary AA supply on the growth performance, faecal dry matter content, apparent faecal nutrient digestibility and N retention in growing pigs.

Health status (H)	HH		LH			P-value		
Dietary AA supply (D)	Adq n = 7	Def n = 6	Adq n = 7	Def n = 5	*SEM*	H	D	H · D
Initial BW[1], kg	26.1	25.5	20.3	19.1	*0.74*	< 0.001	0.31	0.75
Final BW[1,2], kg	29.7	29.2	24.2	22.2	*0.79*	< 0.001	0.23	0.44
ADG[1,3], g/d	505[ab]	536[a]	561[a]	449[b]	*13.1*	0.48	0.07	< 0.01
ADG[1,3], g/(kg BW·d)	18.2[a]	19.6[ab]	25.3c	22.0[b]	*0.73*	< 0.001	0.31	0.02
Gain:feed ratio[2,3]	0.47[a]	0.50[a]	0.63[b]	0.53[a]	*0.016*	< 0.001	0.12	< 0.01
DM content feces, g/kg	300	281	272	252	*5.6*	< 0.01	0.05	0.93
DM digestibility, %	86.5	86.1	85.3	84.8	*0.28*	0.03	0.48	0.88
N digestibility, %	81.8	81.6	78.5	77.4	*0.71*	< 0.01	0.60	0.73
N balance (g/(kg BW·d)								
N intake	0.9	0.9	1.0	1.0	*0.01*			
Fecal N excretion	0.17	0.17	0.21	0.23	*0.01*	< 0.01	0.48	0.59
Urinary N excretion	0.24	0.24	0.19	0.22	*0.01*	< 0.01	0.13	0.12
N retention	0.53	0.53	0.58	0.56	*0.01*	< 0.01	0.26	0.33
N retention / digestible N intake, %	69.0	68.9	75.8	72.0	*0.80*	< 0.001	0.11	0.12

Abbreviations used: ADG, average daily gain; BW, body weight.
[a,b]In case of significant interactions, within a row, means without common superscripts differ ($P < 0.05$).
[1]Initial BW was measured at d 2 and final BW was measured at d 9.
[2]HH-Adq n = 6.
[3]Calculated over a 7-d period.

Table 4.5 Effect of health status and dietary AA supply on the irreversible loss rate (ILR), release from protein breakdown (both in µmol/(kg BW·h)), and pool size (µmol/kg BW) of plasma AA, urea entry rate (µmol/(kg BW·h), urea pool size (µmol/kg BW), and change in ^{13}C enrichment (TTR) of plasma protein in growing pigs[1].

Health status (H)	**HHS**		**LHS**			***P*-value**		
Dietary AA supply (D)	**Adq**	**Def**	**Adq**	**Def**	***SEM***	**H**	**D**	**H · D**
Lys								
Pool size[2]	142	111	139	228	*16.9*	0.15	0.43	0.07
ILR[2]	856	783	941	1096	*45.5*	0.03	0.63	0.19
Breakdown[3]	840	768	924	1079	*45.5*			
Trp								
Pool size	16	14	18	12	*2.4*	0.98	0.50	0.76
ILR	86	68	94	79	*4.1*	0.26	0.06	0.86
Breakdown	84	66	92	77	*4.0*			
Ile								
Pool size	65	34	66	88	*10.7*	0.22	0.84	0.24
ILR	395	325	437	456	*23.9*	0.08	0.59	0.36
Breakdown	383	313	424	443	*23.8*			
Leu								
Pool size	121	56	87	127	*15.2*	0.55	0.67	0.10
ILR	739	561	735	789	*58.7*	0.37	0.62	0.36
Breakdown	715	538	710	764	*58.6*			
Val								
Pool size	175b	92[a]	101[a]	200[b]	*20.4*	0.67	0.84	0.03
ILR	682	583	617	738	*33.6*	0.52	0.88	0.13
Breakdown	666	566	599	720	*33.6*			
Phe								
Pool size	70	24	51	49	*7.5*	0.84	0.12	0.15
ILR	338	226	318	314	*22.4*	0.46	0.22	0.24
Breakdown	326	213	305	301	*22.4*			
Tyr								
Pool size	78	34	48	43	*6.8*	0.41	0.07	0.14
ILR	322	252	298	269	*15.6*	0.92	0.14	0.52
Breakdown	314	244	290	261	*15.6*			
Urea								
Entry rate[2]	702	592	624	693	*34.2*	0.87	0.78	0.24
Pool size[2]	1265	936	1586	1890	*128.3*	0.01	0.96	0.17
^{13}C plasma protein[4]								
Slope*10^8	6.9	7.9	7.1	6.9	*0.36*	0.61	0.61	0.45

[a,b] In case of significant interactions, within a row, means without common superscripts differ ($P < 0.05$).

[1]n = 4-6 for Lys, Ile, Leu, Phe, and Tyr, n = 4-7 for Val, n = 2-6 for Trp, n = 3-5 for urea, n = 5-6 ^{13}C plasma protein.

[2]Calculated from model [2] describing the change in ^{13}C enrichment of plasma AA and $^{15}N_2$-urea after an intravenous injection of seven U-^{13}C labelled AA and $^{15}N_2$-urea.

[3]AA released from protein breakdown was calculated as the difference between ILR of an AA and its dietary intake, using the steady state model (Waterlow, 2006), *i.e.* ILR = protein breakdown + dietary intake = protein synthesis + AA oxidation. Intake was estimated by

multiplying the daily feed intake by the dietary AID content of each AA on a molar basis and dividing this by 24 h. Statistical differences are identical to those of the ILR values, and are therefore not presented.
[4]Calculated from a linear model describing the change in ^{13}C enrichment (TTR) of plasma protein after an intravenous injection of seven U-^{13}C labelled AA and $^{15}N_2$-urea in growing pigs. The slope of ^{13}C enrichment in plasma protein differed from zero ($P < 0.001$).

Discussion

The objective of the present study was to determine the effects of health status and dietary deficiency of Met + Cys, Thr and Trp on whole body N retention and AA utilization in growing pigs.

Contrast in health status

In the present study, the contrast in health status between LHS and HHS pigs was created by selecting two farms at which pigs differed in the presence of antibodies against a number of pathogens. Sows, gilts and growing pigs were monitored on these farms during a period of one year prior to the start of the experiment. At day 2 of the experiment, we observed two times greater serum haptoglobin concentrations, and lower serum albumin concentrations in LHS than in HHS pigs, reflecting a lower health status (Lipperheide *et al.*, 1998; Petersen *et al.*, 2002; Le Floc'h *et al.*, 2006). In line with our findings, haptoglobin was observed to be more responsive to variation in age, stress, sanitary and housing conditions than Pig-MAP and CRP (Heegaardd *et al.*, 2011), and changes in haptoglobin concentrations are more pronounced than Pig-MAP, CRP and albumin in response to natural occurring infections in pigs (Parra *et al.*, 2006). On average, LHS pigs had 51% greater counts of total leukocytes than HHS pigs at day 2 and 29% greater counts at day 9, with the largest quantitative difference in the number of granulocytes, especially neutrophils. An increase in the number of neutrophils is indicative for an infection, possibly of bacterial nature (Zhang *et al.*, 1997; Underhill and Ozinsky, 2002), as neutrophils are short-lived phagocytic cells, acting to kill ingested pathogens (Beutler, 2004). In line with the greater APP concentrations and leukocyte counts, LHS pigs showed abnormalities in selected organs and tissues upon autopsy. Only 3 HHS pigs were studied for autopsy, as the other HHS pigs were used in a subsequent study, but clinical signs of disease were not observed in these 3 HHS pigs. The greater haptoglobin and lower albumin concentrations in serum, the greater leukocyte counts, and the abnormalities observed upon autopsy indicate that LHS pigs had a more activated immune system than HHS pigs. From the start (day 2) to the end (day 9) of the measurement period, the counts of total leukocytes and granulocytes decreased, particularly in LHS pigs. In summary, a clear contrast in the degree of immune system activation between LHS and HHS pigs during the experimental period was observed, although performance parameters indicated that the health status of the LHS pigs was improving. The greater ADG, N retention, and more efficient utilization of digestible N for N-retention in LHS pigs than in HHS pigs indicated possible compensatory effects on these outcomes, likely due to a gradual improvement in

health during the experimental period. The BW at the start of the experiment was lower for LHS pigs than for HHS pigs of similar age. Although the feed intake per kg of metabolic BW did not differ between groups, HHS pigs received a greater absolute amount of feed above maintenance. Nonetheless, ADG was lower in HHS pigs than in LHS pigs, which may be explained by compensatory growth in these pigs.

Effect of health status and dietary treatment on N retention and AA metabolism

The apparent total tract digestibility of DM and N was respectively 1.3 and 3.7% points lower in LHS pigs than in HHS pigs. Reduced digestion and absorption of nutrients can be associated with gastrointestinal tract related diseases, *e.g.* due to intestinal cell damage or increased rate of passage of digesta (Sandberg *et al.*, 2007; Pastorelli *et al.*, 2012). In addition, a parasite infection may have contributed to the observed difference, as observed in the study of Hale *et al.* (1985).

Especially LHS pigs fed the AA adequate diet showed compensatory BW gain upon arrival at the experimental farm, coinciding with an increased efficiency of utilization of digestible N for body protein deposition. These findings are in line with the results of the ILR of AA. In all pigs, the ^{13}C enrichment in plasma AA showed a rapid decline in time, which was accurately described by a double exponential model, as indicated by the goodness of fit. In the present study, the greater ILR for Lys and the tendency for a greater ILR for Ile (P = 0.08) in LHS pigs than in HHS pigs indicate greater use for protein synthesis or greater oxidation of these AA. The greater urea pool size in LHS pigs compared with HHS pigs indicates that the greater ILR for these AA is related to greater oxidation of AA rather than an increase in protein synthesis. Especially in LHS-Def pigs, as the increase in ILR for Lys coincided with a lower ADG. In LHS-Adq pigs, the greater ILR for Lys and numerically higher ILR for Ile were more likely related to greater protein synthesis, as illustrated by a greater ADG, and are consistent with the more efficient use of digested N for protein deposition than in HHS and LHS-Def pigs (+6.8 and +3.8% points, respectively). The labelled AA incorporated in plasma proteins, reflected by the slope of ^{13}C enrichment in plasma protein, was not affected by health status or dietary treatment, indicating that no differences in AA incorporation into plasma proteins were observed between treatments. When expressed relative to the calculated plasma protein pool size, the labelled AA incorporated in plasma proteins was numerically higher in LHS pigs compared to HHS-Adq pigs, but not to HHS-Def pigs.

Feeding a diet deficient in Met + Cys, Thr and Trp was expected to limit protein synthesis, thus reducing the ILR for limiting AA and increasing the ILR of other AA due to greater oxidation of AA that become excessive. Indeed, the dietary treatment Def tended to reduce the ILR for Trp (P = 0.06). It did, however, not lead to greater ILR of the other AA. In HHS pigs, ADG and N retention were unaffected by dietary AA deficiency, indicating that Met + Cys, Thr and Trp were not limiting body protein deposition in HHS pigs. Furthermore, in HHS pigs, ILR, AA released from protein breakdown, and pool size numerically decreased for Lys, Ile, Leu, Val and Phe under Met + Cys, Thr and Trp deficiency. This numerical shift in ILR within HHS pigs likely

reflects reduced protein turnover rates coinciding with decreased oxidative losses of AA in HHS-Def pigs, as the efficiency of N retention was unaffected by AA imbalance in HHS pigs. Coinciding, the 110 µmol/kg BW·h lower urea entry rate and 329 µmol/kg BW lower urea pool size in HHS-Def than HHS-Adq pigs indicate decreased oxidative losses. Within the LHS pigs, the observed compensatory gain in LHS-Adq pigs was associated with a numerical increase in ILR by Met + Cys, Thr and Trp deficiency. This confirms that in the LHS-Adq pigs, the increased N efficiency was related to a reduction in oxidative losses. This suggests that the AA profile of the Def dietary treatment limited body protein deposition in LHS pigs but not in HHS pigs. The gradual improvement in performance of especially LHS-Adq pigs, however, may in part have masked the effect of health status on N retention and AA utilization in the current study.

The observed tendency (P = 0.09) for a greater serum haptoglobin concentration in LHS-Def pigs than in LHS-Adq pigs indicate that the dietary AA supply was certainly not limiting the production of APP in LHS-Def pigs. Furthermore, it is possible that the deficient dietary AA a pro-inflammatory response as described below. These findings imply that a deficient dietary supply of Met + Cys, Thr and Trp increased the competition for AA between body protein deposition and the synthesis of proteins associated with immune system activation in LHS pigs.

Effect of dietary AA supply on the immune response

In the present study we observed a 30% greater monocyte count in Def pigs than in Adq pigs, with the difference being most pronounced in LHS pigs. Monocytes are the most common cells to initiate the acute phase response by releasing cytokines (Baumann and Gauldie, 1994). In line, haptoglobin concentration and the haptoglobin to albumin ratio tended to be higher in Def pigs, particularly in LHS (interaction health · diet; P = 0.09 and 0.07, respectively) . Dietary AA supply has previously been associated with changes in the immune system (Grimble, 2001; Li *et al.*, 2007; Calder and Yacoob, 2012). The systemic release of pro-inflammatory cytokines, for example, is inhibited by dietary Trp supplementation to pigs with experimentally induced colitis (Housemann *et al.*, 1973). In humans, reducing protein intake from 1.4 to a marginally adequate 0.6 g/kg BW/day for 7 days induced a low grade inflammatory response, as indicated by increased plasma concentrations of interleukin-6 and synthesis of positive APP (haptoglobin and fibrinogen), while the albumin synthesis decreased (Jackson *et al.*, 2001). Similarly, Le Floc'h *et al.* (2008) observed numerically greater plasma haptoglobin concentrations in Trp-deficient pigs than in Trp supplemented pigs with an experimentally induced lung inflammation. Furthermore, a deficient Trp supply in pigs was associated with greater relative lung weights (Le Floc'h *et al.*, 2008), and with greater intestinal damage in experimentally induced colitis (Kim *et al.*, 2010).

In summary, LHS pigs had a lower health status, especially at the start of the experiment and a reduced total tract DM and N digestibility compared to HHS pigs. LHS pigs fed the AA adequate, and to a lesser extent the AA deficient diet, showed

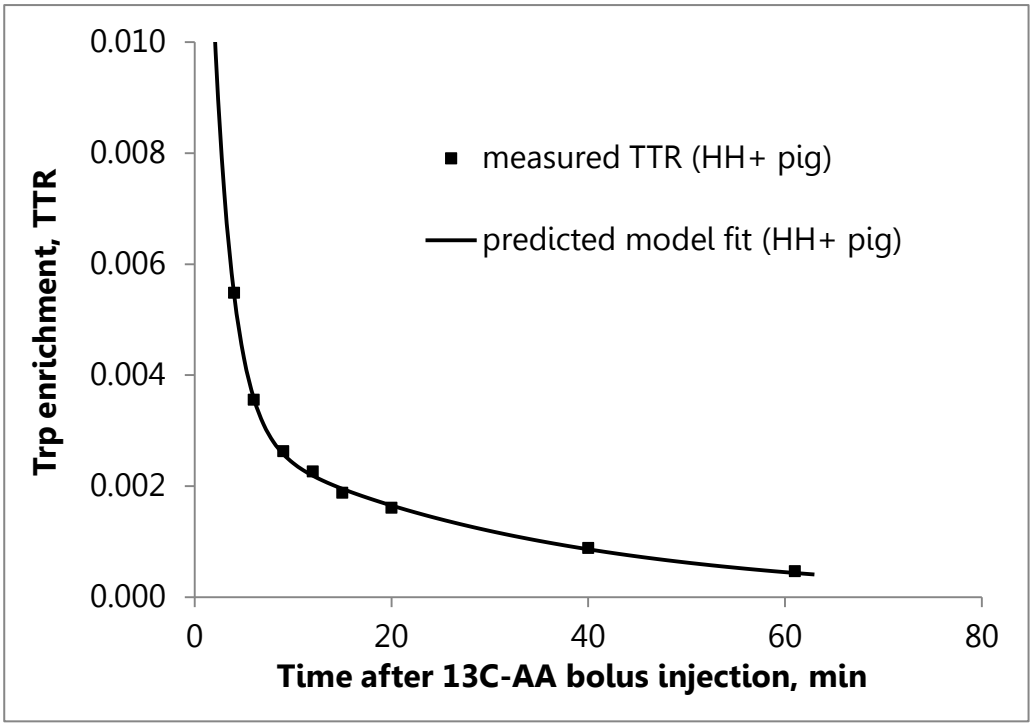
compensatory BW gain after arrival at the experimental farm, coinciding with greater N retention and an increased efficiency of digestible N utilization for growth when compared with HHS pigs. A reduced health status increased ILR for Lys and tended to do so for Ile. Changes in Lys, Val and urea pool sizes support the observation that the increase in ILR of Lys under LHS conditions relates to oxidation in pigs fed the AA deficient diet, whereas it relates to synthesis in pigs fed the AA adequate diet. Feeding Met + Cys, Thr and Trp deficient diets increased monocyte counts and tended to do so for haptoglobin concentrations, particularly in LHS pigs. This illustrates how the competition for AA between synthesis of proteins associated with immune system activation and body protein deposition is enlarged when dietary supply of Met + Cys, Thr and Trp is limiting in pigs during and following a period of low health.

Supplemental Material

Supplemental Table 4.1 Serological results of the health monitoring program of the High Health status (HHS) and Low Health status (LHS) farm[1].

Characteristics	High Health status farm	Low Health status farm
Seronegative to antibodies against:	PRRSV Swine Vesicular Disease *MYC* *APP* (type 1, 2, 5, 9, 11) *BRA* *Pasteurella multocida*, *Brucella suis*	*Pasteurella multocida* *Heamophilus parasuis*
Seropositive to antibodies against:	Influenza H1N1 *Lawsonia intracellularis* *Salmonella*	PRRSV Influenza H1N1 and H3N2 *MYC* *APP* (type 1, 2, 5) *Lawsonia intracellularis* Circo IgG and IgM

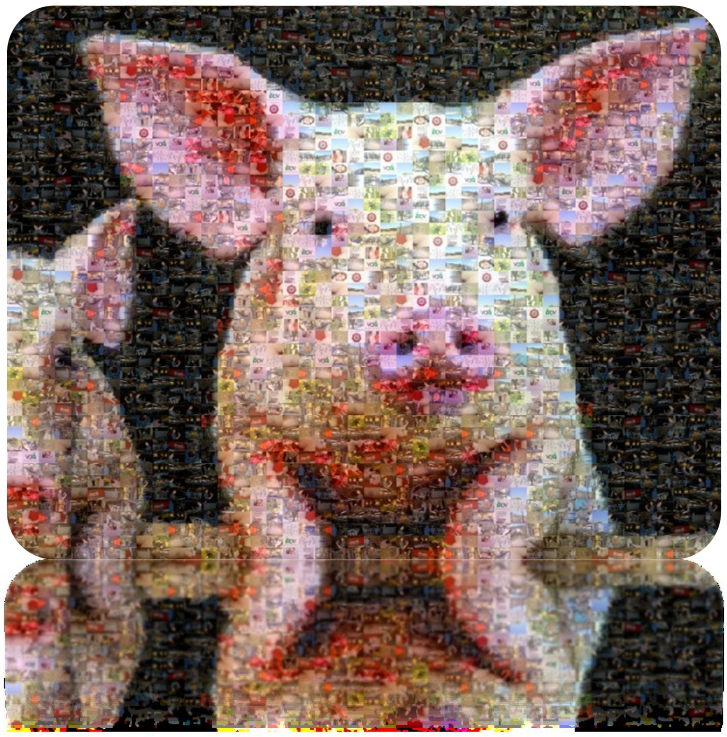
Abbreviations used: APP, *Actinobacillus pleuropneumoniae*; BRA, *Brachyspira dysentery*; MYC, *Mycoplasma hypneumoniae*; PRRSV, Porcine reproductive and respiratory syndrome virus.
[1]The serological monitoring program (Topigs, The Netherlands) included minimum quarterly monitoring of sows, gilts and growing pigs during a period of one year prior to the start of the experiment.



Supplemental Figure 4.1 Example: Measured (■) and predicted ^{13}C-enrichment in plasma Trp (TTR) with a double exponential model ($E(t) = a1 \cdot \exp(b1 \cdot t) + a2 \cdot \exp(b2 \cdot t)$) after injection of the U-^{13}C labelled AA mixture of one single pig.

Chapter 5

Induced lung inflammation and dietary protein supply affect nitrogen retention and amino acid metabolism in growing pigs



Esther Kampman - van de Hoek[ab], Panagiotis Sakkas[b], Walter J. J. Gerrits[b], Joost J. G. C. van den Borne[b], Carola M. C. van der Peet-Schwering[a], and Alfons J. M. Jansman[a]

British Journal of Nutrition (2015) **113**, 414-425.

[a]WUR Livestock Research, PO Box 338, 6700 AH Wageningen, The Netherlands
[b]Animal Nutrition Group, Wageningen University, PO Box 338, 6700 AH Wageningen, The Netherlands

Abstract

It is hypothesised that during immune system activation there is a competition for amino acids (AA) between body protein deposition and immune system functioning. The aim of the present study was to quantify the effect of immune system activation on N retention and AA metabolism in growing pigs, depending on dietary protein supply. A total of sixteen barrows received an adequate (A) or restricted (R) amount of dietary protein, and were challenged at day 0 with intravenous (*i.v.*) complete Freund's adjuvant (CFA). At day -5, 3 and 8, irreversible loss rate (ILR) of 8 AA was determined. CFA successfully activated the immune system, indicated by 2 to 4 fold increases in serum concentrations of acute phase proteins (APP). Pre-challenge C-reactive protein concentrations were lower ($P < 0.05$) and pre- and post-challenge albumin tended to be lower in R-pigs. These findings indicate that a restricted protein supply can limit the acute phase response. CFA increased urinary N losses ($P = 0.04$) and tended to reduce N retention in A-pigs, but not in R-pigs ($P = 0.07$). ILR for Val was lower ($P = 0.05$) at day 8 than at day 3 post-challenge. ILR of most AA, except for Trp, were strongly affected by dietary protein supply and positively correlated to N retention. Correlations between ILR and APP indices were absent or negative, indicating that changes in AA utilisation for APP synthesis were either not substantial, or more likely, outweighed by a decrease in muscle protein synthesis during immune system activation in growing pigs.

Keywords: Inflammation; Amino acid metabolism; Dietary amino acid supply; Growing pigs

Introduction

During immune system activation in animals, nutrients are redistributed from anabolic and maintenance processes towards processes involved in immunity and disease resistance (Klasing and Johnstone, 1991; Spurlock, 1997). A cascade of cytokine-induced metabolic alterations occur, including anorexia, increased breakdown and decreased synthesis of skeletal muscle protein, increased hepatic acute phase protein (APP) synthesis, and increased deamination of gluconeogenic amino acids (AA) (Klasing and Johnstone, 1991; Lochmiller and Deerenberg, 2000; Le Floc'h *et al.*, 2004). The acute phase response is the early innate immune response to injury, trauma or infection, and increases serum concentrations of positive APP while decreasing concentrations of negative APP (Baumann and Gauldie, 1994). Synthesis of positive APP during an acute phase response is considered to be nutritionally more costly than the adaptive response to inflammation, *i.e.* leukocyte proliferation and antibody production (Iseri and Klasing, 2013). Reeds *et al.* (1994) calculated that an APP response increases the demand for aromatic AA in particular. For the synthesis of APP, AA are provided either from dietary protein or from breakdown of skeletal muscle protein. The AA composition of APP differs, however, largely from that of muscle protein (Reeds *et al.*, 1994), and from commercial diets, which are formulated mainly to enhance muscle protein deposition. It is hypothesised that, as a consequence there can be an imbalance in available AA for body protein deposition, leading to increased oxidation of AA and N loss, which is close to the quantitative N loss observed in uncomplicated trauma (Reeds and Jahoor, 1994). Moreover, the cytokine induced metabolic change after immune system activation generally results in increased breakdown and decreased synthesis of skeletal muscle protein (Breuille *et al.*, 1999; Zamir *et al.*, 1992).

In pigs, immune system activation, by continuous exposure to major vectors of antigen transmission (Williams *et al.*, 1997), or by *i.m.* lipopolysaccharide (LPS) injection (Daiwen *et al.*, 2008), reduces feed intake, body weight (BW) gain and N retention. Recent studies in pigs revealed that immune system activation by *i.m.* LPS administration increases the optimal dietary Met to Met + Cys ratio (Litvak *et al.*, 2013b), and reduces the efficiency of Trp utilisation for body protein deposition (de Ridder *et al.*, 2012). These findings indicate that the utilisation for AA in growing pigs may change due to variation in health status. However, quantitative information about the effect of immune system activation on the utilisation for AA is lacking, and measurements on changes in responses of multiple AA simultaneously to immune system activation are largely absent. Alterations in AA metabolism, *e.g.* an increased protein synthesis rate, can occur without concomitant changes in plasma AA concentrations or pool size, as plasma AA concentrations can be maintained when AA fluxes change by changes in dietary protein intake, breakdown and synthesis of body protein and oxidation of AA (Waterlow, 2006). The irreversible loss rate (ILR) of AA, reflects the amount of free AA that disappears per unit of time from the plasma pool for protein synthesis and oxidation. The combination of ILR measurements with N balance and pool size measurements can provide insight into the metabolic changes in

multiple AA simultaneously. It is hypothesized that an increase in blood APP during immune system activation affects the utilisation of AA, associated with an increased incorporation of, in particular aromatic, AA into APP.

In the present study, intravenous (*i.v.*) administration of complete Freund's adjuvant (CFA), that has been previously shown to induce chronic lung inflammation in pigs (Melchior *et al.*, 2004; Le Floc'h *et al.*, 2008), was used to activate the immune system. It is hypothesized that the effect of a CFA challenge on protein metabolism is more pronounced under conditions of a marginal dietary protein supply, which would increase the competition for indispensable AA used for immune system functioning and for protein deposition in muscle as a main determinant of the animal's growth. In addition, there is increasing evidence that the dietary protein or AA supply can affect the inflammatory response during immune system activation (Grimble *et al.*, 1992; Jahoor *et al.*, 1999; Li *et al.*, 1999; Grimble, 2001; Li *et al.*, 2007; Le Floc'h *et al.*, 2008; Le Floc'h *et al.*, 2009; Calder and Yaqoob, 2012). A Trp deficient diet, for instance, was suggested to deteriorate the immune response to CFA, as indicated by increased IDO activity in lungs and heart, and increased lung weight (Le Floc'h *et al.*, 2008) in contrast to a Trp supplemented diet. As IDO is induced by cytokines (Moffet and Namboodiri, 2003), its activity is associated with the degree of immune system activation. In another study, the addition of Cys to a protein deficient diet increased liver weight and hepatic glutathione concentrations, following an intraperitoneal injection of TNF-α in rats (Grimble *et al.*, 1992). The latter authors suggested that in that study Cys supplementation improved the immune response following TNF-α administration, enabling a full metabolic response to cytokines that improves the ability to maintain antioxidant defences. Although it is debatable whether a change in immunological response is beneficial or not, these findings show that the dietary protein or AA supply can affect the inflammatory response during immune system activation. The aim of the present study was to quantify the effect of immune system activation on N retention and AA metabolism in growing pigs, depending on dietary protein supply.

Material and methods

Animals and treatments

The experiment was approved by the Animal Experimental Committee of Wageningen UR Livestock Research. Sixteen barrows (Dutch Landrace · York) with an initial BW of 28.5 ± 0.5 kg were individually housed in metabolism cages (1.3 · 1.3 m) at a room temperature ranging between 18 and 22°C. Based on litter and BW, pigs were allocated to one of two treatment groups receiving either an adequate (A) or restricted (R, 70% of A) dietary protein supply at a similar daily supply of other nutrients. To this end, a basal mixture was created without protein sources. The A diet included the basal mixture with the additional protein sources casein, wheat gluten meal, soy protein isolate, and potato protein, and met the requirements for essential AA for growing pigs in the range of 35 to 45 kg BW (CVB, 2008) (Table 5.1). The R diet included the basal mixture to which 70% of the quantities of additional protein sources (casein, wheat

gluten meal, soy protein isolate, potato protein) were added compared to the quantities included in the A diet. In order to supply all pigs with the same amount of basal mixture, relative to their metabolic BW, the feed allowance of pigs assigned to the R diet was 94.3% of that of pigs receiving the A diet. The experimental diets were provided in mash form and mixed with water using a feed to water ratio of 1 : 3. Pigs were fed at 0700 and 1530 h in equal amounts at 2.7 times the energy requirements for maintenance (M; 458 kJ ME/(kg $BW^{0.75}$/day); ARC, 1981). Feed refusals were collected 30 min after feeding.

Table 5.1 Composition of the experimental diets (as-fed basis).

	Adequate protein diet	Restricted protein diet
Ingredient composition, g/kg		
Basal mixture[1]		
Wheat starch	250.0	265.2
Pregelatinized potato starch	240.1	254.7
Oat hulls	100.0	106.1
Dextrose	100.0	106.1
Beet pulp	50.0	53.0
Soybean oil	30.0	31.8
Calcium carbonate	15.5	16.4
Monocalcium phosphate	11.6	12.3
Potassium carbonate	6.2	6.6
Sodium chloride	3.9	4.1
Vitamin and mineral premix[2]	2.0	2.1
Protein containing ingredients		
Soy protein isolate	90.0	66.8
Casein	65.0	48.2
Wheat gluten meal	29.7	22.1
Potato protein[3]	5.3	3.9
DL-Met	0.9	0.6
Calculated composition, g/kg[4]		
DM	927	927
NE, MJ/kg[5]	10.55	10.63
Crude protein	170	129
Crude ash	51	52
Crude fat	36	37
Crude fiber	37	39
AID Lys	9.2	6.9
AID Met	3.9	2.9
AID Met + Cys	5.5	4.1
AID Thr	5.5	4.1
AID Trp	1.8	1.3
AID Ile	7.2	5.4
AID His	4.1	3.1
AID Leu	13.1	9.8
AID Phe	8.1	6.1
AID Val	8.1	6.0
Analysed composition, g/kg		
Crude protein	182	137
Total Lys	11.2	8.5
Total Met	4.1	3.0
Total Met + Cys	5.8	4.4
Total Thr	6.7	5.2
Total Trp	2.0	1.6
Total Ile	8.2	6.3

Total His	4.6	3.6
Total Leu	14.9	11.4
Total Phe	9.2	7.1
Total Val	9.7	7.4

Abbreviations used: AID, apparent ileal digestible; NE, net energy.
[1]Two levels of dietary protein supply (adequate (A) or restricted (R, 70% of A) were used in the study, at a similar daily supply of other nutrients. In the restricted protein supply, the proportion of protein containing ingredients in the diet was reduced with 30% relative to the proportion in the adequate protein diet. In order to supply all pigs, relative to their metabolic BW, with the same amount of basal mixture, the feed allowance of pigs fed the R diet was 94.3% of those fed the A diet.
[2]Vitamin and mineral premix provided per kg of adequate or restricted diet, respectively: 2.4 or 2.5 mg of vitamin A; 50 or 52,5 µg of cholecalciferol; 14.7 or 15.7 mg of vitamin E; 1.5 or 1.6 mg of vitamin K; 1.0 or 1.1 mg of thiamin; 4.0 or 4.2 mg of riboflavin; 12.0 or 12.6 mg of pantothenic acid; 20.0 or 21.0 mg of niacin; 20.0 or 21.0 µg of vitamin B_{12}; 0.20 or 0.21 mg of folate; 1.0 or 1.1 mg of vitamin B_6; 100 or 105 mg of choline chloride; 100 or 105 mg of Fe as $FeSO_4$; 10.0 or 10.5 mg of Cu as $CuSO_4 \cdot 5H_2O$; 65.0 or 68.3 mg of Zn as ZnO; 30.0 or 31.5 mg of Mn as MnO; 0.15 or 0.16 mg of Co as $CoSO_4$; 0.75 or 0.79 mg of K as KI; and 0.30 or 0.31 mg of Se as Na-selenite.
[3]Protastar®, Avebe Feed, Veendam, The Netherlands.
[4]Unless indicated otherwise.
[5]NE was calculated based on CVB (2004).

At day -16 or -14 before the start of immune system activation, pigs were surgically fitted with a jugular vein and a carotid artery catheter for blood collection and injection of a mixture of U-^{13}C labelled AA, respectively. Neopen (Neomycine 5 mg/kg BW and Procaïne benzylpenicilline 10,000 IE/kg BW; Intervet, Boxmeer, The Netherlands) was given intramuscular (*i.m.*) 1 day before surgery, at surgery and 1 day after surgery. Flunixine (Fynadine 2.2 mg/kg BW; Schering-Plough, Heist-op-den-Berg, Belgium) was given *i.m.* at surgery and for 2 day after surgery. A timeline of the experiment is shown in Figure 5.1 with day 0 being the start of immune system activation by *i.v.* administration of CFA (F5881, Sigma-Aldrich, St. Louis, MO, USA). CFA is a mineral oil containing 1 mg dead *Mycobacterium tuberculosis* cells per mL. The dose of CFA administered per pig was 0.2 mL/kg BW, diluted with saline in a ratio of 1 : 2. The dose was spread over four equal sub-doses, of which two were infused at day 0 and two at day 1, in the morning between 0915 and 1030 h, and in the afternoon between 1515 to 1630 h. Eight pigs did not receive the fourth sub-dose of CFA, as clinical observations after the first infusions on 8 pigs showed a more severe response, *i.e.* greater and persistent feed refusals, greater increase in respiratory rhythm, than expected based on a preliminary study (unpublished results).

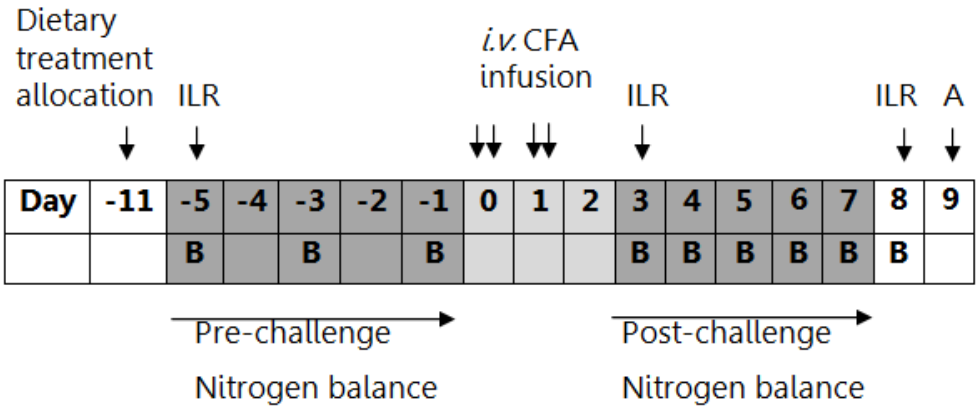


Figure 5.1 Timeline of the experimental period. A, autopsy; BS, blood sampling; ILR, irreversible loss rate measurement by the injection of the U-^{13}C-labelled amino acid mixture; *i.v.,* intravenous; CFA, complete Freund's adjuvant.

Immunological response parameters

At day -5, -3, -1, 3, 4, 5, 6, 7 and 8, blood samples were collected into serum tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria) and allowed to clot for 1 h at room temperature. Serum was collected after centrifugation for 10 min at 1,800 · *g* and was stored at -20°C pending analyses of albumin (Randox Bromocresol Green assay, cat. no. AB 362), C-reactive protein (CRP, ELISA, Reactivlab Limited, Glasgow, Scotland), haptoglobin (Tridelta Phase Haptoglobin Assay, cat. no. TP-801), pig major acute phase protein (pigMAP, ELISA, Reactivlab Limited, Glasgow, Scotland), and total protein (Biuret reaction; Doumas *et al.*, 1981). At day -5, 0, 1, 3, 5 and 8, blood samples were collected into EDTA tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria) and analysed for the number of total white blood cells (WBC). At day 9, all pigs were euthanized and autopsy was performed by an experienced pathologist. Autopsy observations included assessment of body condition, visual inspection for abnormalities of lung, spleen, stomach, small intestine, large intestine, kidney, liver, heart, and mesenteric lymph nodes, determination of lung weight, and histological evaluation of lungs, liver, tracheobronchial lymph nodes, and kidneys.

Nitrogen balance

Pigs were equipped with a Velcro support system to allow separate collection of faeces (Van Kleef *et al.*, 1994) and urine. Faeces and urine were collected quantitatively from each pig during two periods of 5 subsequent days each, *i.e.* in the pre- and post-challenge period (Figure 5.1). Faeces were stored at -20°C pending analysis. Urine was collected via funnels, which were sprayed with an acetic acid buffer (sodium acetate 0.08 M, formic acid 0.025 M, and acetic acid 0.013 M) to prevent evaporation of NH_3, into buckets containing sulphuric acid (4.5 M), to maintain a pH < 3 for conservation. Urine was collected daily from the buckets, weighed, sampled and stored at -20°C pending analysis. Nitrogen in urine and fresh faeces was analysed using the

Kjeldahl method (ISO, 2005a). DM content of faeces was determined by drying at 103°C (ISO, 1999).

Amino acid metabolism

At pre-challenge (day -5), early post-challenge (day 3) and late post-challenge (day 8), the fluxes of plasma Lys, Trp, Met, Ile, Leu, Val, Phe, and Tyr were studied by measuring the change in plasma isotopic enrichment of individual AA in time after an *i.v.* U-^{13}C labelled bolus of these AA. To create a steady state in dietary AA supply at the day of injection of the U-^{13}C labelled AA mixture and frequent blood sampling, the daily feed allowance was spread over ten equal meals. Two meals were fed at 0730 h, followed by hourly meals from 0910 h until 1610 h. At 1230 h, a mixture of 11 U-^{13}C labelled AA (97 to 99 atom percent ^{13}C, Cambridge Isotope Laboratories, Andover, MA, USA) was injected. The composition of the mixture (mg/g saline) was L-Lys, 0.17; L-Thr, 0.18; L-Trp, 0.07; L-Met, 0.06; L-Cys, 0.04; L-Ile, 0.16; L-Leu, 0.16; L-Val, 0.19; L-Phe, 0.13; L-Tyr, 0.16, and L-His, 0.08. The mixture was injected as a bolus (0.50 g/kg BW; 0.25 mL/s) in the carotid artery. If the carotid artery catheter was blocked, the mixture was injected in the jugular vein. Blood samples (4 mL each) were collected from the jugular vein and transferred into tubes containing lithium heparin (Vacuette, Greiner Bio-One, Kremsmünster, Austria) at 10 min before injection of the U-^{13}C labelled AA mixture and at 3, 5, 7, 9, 11, 15, 25, 45, 80, and 120 min after injection. Tubes were immediately placed on ice and centrifuged for 10 min at 2,000 · *g* at 4°C, after which plasma was collected and stored at -20°C pending analysis. In each blood sample, ^{13}C enrichment was measured in plasma Lys, Trp, Met, Ile, Leu, Val, Phe, and Tyr as ethyl chloroformate ester (ECF, Merck Schuchardt OHG, Hohenbrunn, Germany) derivatives by GC-combustion-isotope ratio MS (isotope ratio MS, Delta V Advantage, Thermo Scientific, Bremen, Germany; GC Trace Ultra, Thermo Scientific, Milan, Italy (column no. CP8982, VF-17ms 30 m · 0.25 mm, film 0.25 µm, Agilent Technologies, Amstelveen, The Netherlands); and combustion , Combustion III, Thermo Scientific, Bremen, Germany), as adapted from Huang *et al.* (2011). Briefly, 20 µL hydrogen chloride (1 N) and 200 µL Dowex ion exchange resin (Ag 50W-X8 H+ form, 200-400 mesh, Dow Chemical Company, Edegem, Belgium) was added to 180 µL plasma and eluted with 0.7 mL ammonium hydroxide (6 N) to isolate free plasma AA. The supernatant was evaporated with a centrifugal concentrator (Jouan RC 1022, Thermo Scientific, Marietta, OH, USA) under vacuum at room temperature. Derivatisation was performed by adding 140 µL ethanol-pyridine (4 : 1 by volume) and 20 µL ECF to the dry supernatant. Derivates were extracted by adding 4 · 200 µL hexane-dichloromethane-ECF (50 : 50 : 1 by volume) and the supernatant was dried in a vial under N_2 gas at room temperature. After dissolving in 50 µL ethyl acetate, the sample was injected in triplicate into the GC. The ^{13}C enrichment of His could not be successfully analysed due to high losses of His during the derivatization step. ^{13}C enrichment in Thr and Cys could not be determined with the current procedure, and additional derivatization steps would be required for their measurement.

Calculations

Dietary N intake, faecal and urinary N excretion and whole body N retention were expressed relative to metabolic BW (kg $BW^{0.75}$). Relative lung weight was calculated as a percentage of BW. ^{13}C enrichment in plasma AA was expressed as tracer-to-tracee ratio (TTR). To calculate a change in TTR in time, for each AA the background enrichment (obtained from plasma samples taken before injection of the U-^{13}C labelled AA mixture) was subtracted from the ^{13}C enrichment in samples after injection.

ILR of AA from plasma was calculated from the change in ^{13}C enrichment of plasma AA after the *i.v.* administered bolus of U-^{13}C labelled AA using the model and calculations as described by Holtrop *et al.* (2004). The following assumptions were made: there is a physiological steady state during the measurement, *i.e.* a constant size of the plasma AA pool, so that the inflow of AA into the plasma pool equals the outflow from the plasma pool (Waterlow, 2006); the tracer transfers along with the tracee between compartments with a constant fractional rate (Waterlow, 2006); the ILR for an AA occurs as an output from the plasma pool, and only by incorporation of the AA into synthesised protein or by loss of AA via oxidation (Reeds *et al.*, 1980). Finally, once the tracer has entered the body protein pool there is no recycling of the tracer into the plasma pool, as the whole body protein pool is a large pool with a low turnover rate compared to the plasma pool (Waterlow, 2006). A double exponential model was fitted to the ^{13}C enrichment of individual plasma AA after administration of the bolus injection:

$$E(t) = a_1 \exp(b_1 t) + a_2 \exp(b_2 t) \quad [1]$$

where E(t) is the predicted ^{13}C enrichment in plasma AA (TTR) at time t (min), and a_1, b_1, a_2, and b_2 are parameter estimates from which the ILR (µmol/(kg BW·h)) was calculated:

$$ILR = d / (a_1 / b_1 + a_2 / b_2)\ 60 \quad [2]$$

where d is the dose of administered U-^{13}C labelled AA (µmol/kg BW).

For each AA and pig the pool size, *i.e.* the amount of AA in the pool (µmol/kg BW), was calculated as:

$$\text{Pool size} = d0 / (a1 + a2) \quad [3]$$

An ILR index was calculated for each pig and time point as the ILR at day -5, 3 or 8 divided by the mean ILR at day -5, 3 and 8 of that particular pig, multiplied by 100. This index indicates the change in ILR within animals as affected by the challenge.

AA released from protein breakdown was calculated as the difference between ILR and dietary intake, using the steady state model of Waterlow (2006), *i.e.* ILR = protein breakdown + dietary intake = protein synthesis + AA oxidation. Intake was estimated by multiplying the feed intake by the dietary AID content of each AA and divided by 24 h and the molar mass.

Two indices were calculated from serum concentrations of APP: a nutritional acute phase index (NAPI), and a health status acute phase index (HAPI). NAPI was considered

to be associated with the nutritional costs of APP synthesis, implying that the half-lives of the APP should be taken into account. The half-life of a positive APP is considered to be inversely related to the requirements for AA for APP synthesis. To amplify the nutritional costs of an APP response, all measured positive APP are divided by the half-life (CRP 19 h (Vigushin *et al.*, 1993); haptoglobin 132 h (Dobryszycka *et al.*, 1979); pigMAP 132 h, the latter value assumed to be similar as for haptoglobin, based on the response pattern (Petersen, 2004).

$$\text{NAPI} = (\text{pigMAP (g/L)} / \text{half-life}) + (\text{CRP (g/L)} / \text{half-life}) + (\text{haptoglobin (g/L)} / \text{half-life}) \quad [4]$$

HAPI was calculated as the sum of positive APP indices divided by the index for albumin as a negative APP, in which each index reflects the change in APP within a pig relative to the mean APP at day -5, 3 and 8 of that pig. HAPI was considered as a general indicator of health status. By including the indices for positive and negative APP in HAPI, the range in values is amplified (Toussaint *et al.*, 1995; Gruys, 2005).

$$\text{HAPI} = \frac{\text{CRP index} + \text{haptoglobin index} + \text{pigMAP index}}{\text{albumin index}} \quad [5]$$

where the APP index (*e.g.* CRP index) was calculated for each pig and time point as the APP concentration at day -5, 3 or 8 divided by the mean APP concentration at day -5, 3 and 8 of that particular pig, multiplied by 100.

Statistical analysis

Pig was considered as the experimental unit. Effects of dietary treatment and collection day or period on WBC, N balance measures, ILR, AA release in plasma from protein breakdown, and AA plasma pool size were analysed with a mixed model with collection day, or period, taken as repeated measures. Fixed effects also included the interaction between dietary treatment and collection day, or period. Effects were analysed by pairwise comparisons using Tukey-Kramer adjustment. A covariance structure was chosen based on the lowest value for the Akaike and Bayesian information criteria. The effect of dietary treatment, collection day and the interaction between both on APP and total protein serum concentrations were analysed separately per period (pre- and post-challenge) with a mixed model with collection day taken as repeated measure. For the post-challenge APP serum concentrations, the mean of pre-challenge serum APP concentrations (day -5, -3 and -1) was used a covariate. The effect of dietary treatment on relative lung weight was analysed by ANOVA. To associate a change in AA utilisation with a change in APP concentrations or N balance, the correlation between ILR index for AA and NAPI or HAPI, and the correlation between N retention and ILR or ILR index were determined using a Pearson correlation

analysis. To distinguish between the two days post-challenge (day 3 and 8), the correlation analyses were performed separately in two parts, *i.e.* including data of pre- and day 3 post-challenge, and including data of pre- and day 8 post-challenge.

The normality of distribution of studentized residuals was assessed. Data on pigMAP and CRP were log transformed to obtain normal distribution of model residuals. All statistical procedures were conducted in SAS (SAS Inst. Inc., Cary, NC, USA). Values are presented as means ± (pooled) SEM, and effects were considered significant at $P \leq 0.05$.

The goodness of fit of the double exponential model used to fit the ^{13}C enrichment of individual plasma AA was assessed by the mean square prediction error (MSPE). The root MSPE was scaled to the observed mean (mean prediction error) and the correlation between predicted and observed values was calculated. Errors due to overall bias, due to deviation of the regression slope from unity, and due to random variation were calculated (Bibby and Toutenburg, 1977). Enrichment data of some AA were excluded due to unrealistic parameter estimation and concomitant unrealistic ILR.

Results

Four pigs had feed refusals that exceeded 10% of their daily allowance during the post-challenge measurement period. Data from these pigs were excluded from the experiment. One pig was excluded from the experiment due to illness occurring before the start of the N balance measurements.

Immunological response

Pre-challenge, serum CRP concentrations were lower ($P = 0.02$) in R-pigs than in A-pigs. In the pre-challenge period, dietary protein supply did not affect serum concentrations of haptoglobin, pigMAP, and total protein. In the pre- and post-challenge period, R-pigs tended to have lower ($P = 0.09$) serum albumin concentrations than A-pigs. In the post-challenge period, dietary protein supply did not affect serum concentrations of CRP, haptoglobin, pigMAP, and total protein.

In the pre-challenge period, serum albumin concentrations were higher ($P = 0.007$) at day -5 than at day -3 and day -1 (Figure 5.2). In the post-challenge period, collection day affected serum concentrations of all APP and total protein. Serum concentrations of CRP peaked at day 5 post-challenge ($P < 0.001$) and declined thereafter. Serum concentrations of haptoglobin peaked at day 3 ($P < 0.001$) and declined thereafter. Serum concentrations of pigMAP peaked at day 3 ($P < 0.001$) and declined thereafter (Figure 5.2). Serum concentrations of albumin ($P = 0.001$) and total protein ($P = 0.002$) showed a drop at day 4.

WBC counts were unaffected by dietary protein supply. The WBC count was lower at day 1 post-challenge ($P = 0.03$) than at day -5 pre-challenge and day 8 post-challenge

(Figure 5.2). The WBC count was lower at day 0 pre-challenge (P = 0.03) than at day 8 post-challenge (Figure 5.2).

Autopsy results revealed that *i.v.* administered CFA induced a moderate to severe granulomatous interstitial pneumonia. The relative lung weight was 1.52 ± 0.10% of BW at day 9 post-challenge, and was unaffected by dietary protein supply. Six pigs showed signs of lymphohisticytic focal hepatitis, and one pig showed signs of lymphocytosis. In one pig, sinushistiocytosis was observed in the tracheobronchial lymph nodes. No abnormalities were found in other organs.

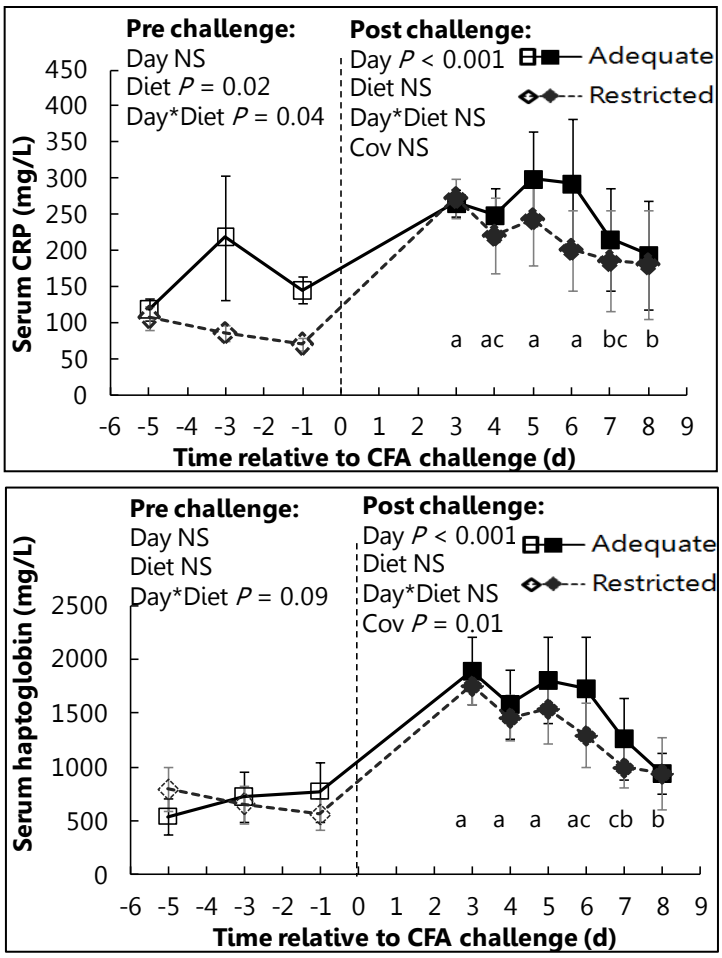


Figure 5.2 Effect of CFA challenge and dietary protein supply (Adequate, A or Restricted, R) on serum acute phase proteins, serum total protein concentrations, and white blood count (WBC) in growing pigs (n = 11). Open and closed symbols indicate pre- or post-challenge measures, respectively. Within the pre- or post-challenge period, means of each day without a common letter differ (referring to the day effect). Cov, covariate; in the analysis of post-challenge APP serum concentrations, the mean of pre-challenge APP concentrations (day -5, -3 and -1) was used a covariate.

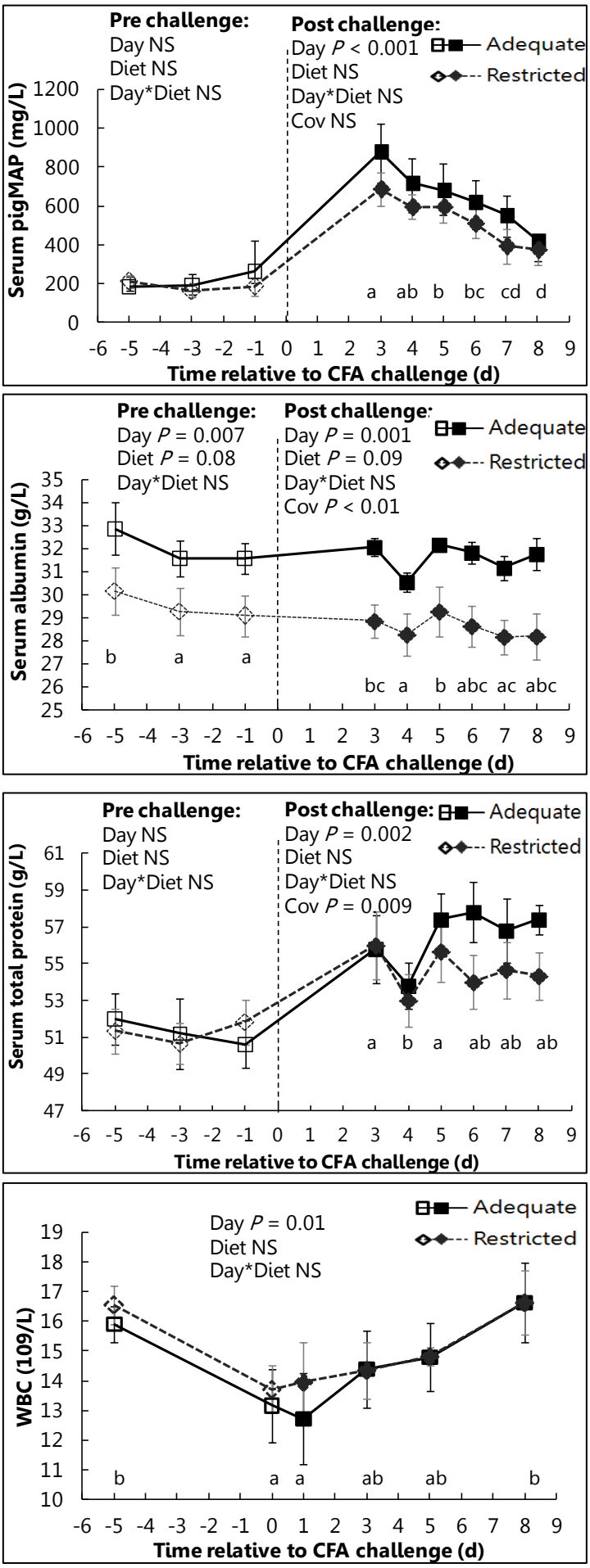


Figure 5.2
(continued)

Performance and nitrogen retention

Dietary protein supply did not affect faecal N excretion. Total tract N digestibility was greater in A-pigs than R-pigs (P = 0.001). Urinary N excretion was greater in A-pigs than in R-pigs (P < 0.001), and N retention was 20% lower in R-pigs than in A-pigs (P < 0.001). N utilisation for retention was greater in R-pigs (P = 0.02) than in A-pigs (Table 5.2). CFA challenge did not affect N intake, faecal N excretion, and apparent total tract N digestibility (Table 5.2). Urinary N excretion was greater post-challenge than pre-challenge (P = 0.04). N retention (P = 0.07) and N utilisation for retention (P = 0.07) tended to be greater in the pre- than in the post-challenge period.

Amino acid metabolism

The double exponential model accurately described the decrease in ^{13}C enrichment of individual plasma AA after injection of the bolus with ^{13}C AA. The average root MSPE of the studied AA ranged between 4.8 and 6.9%, with > 95% of the prediction error attributable to random variation.

R-pigs had a lower ILR for Lys (P = 0.02), Met (P = 0.03), Ile (P = 0.05), Val (P < 0.01), and Tyr (P < 0.01) than A-pigs, and tended to have a lower ILR for Phe (P = 0.09). ILR for Leu and Trp was not affected by dietary protein supply. R-pigs had a lower Lys (P = 0.03), Val (P = 0.02), and Tyr (P = 0.03) release from protein breakdown than A-pigs, and the Met release from protein breakdown tended to be lower in R-pigs (P = 0.06). The Trp, Ile, Leu and Phe release from protein breakdown was not affected by dietary protein supply. Lys pool size tended (P = 0.08) to be lower in R-pigs than in A-pigs (Table 5.3).

Table 5.2 Effect of CFA challenge and dietary protein supply (Adequate, A or Restricted, R) on growth performance and N balance in growing pigs (mean values with the pooled SEM).

Diet	Adequate (A)		Restricted (R)			*P*-value		
Challenge	**Pre**	**Post**	**Pre**	**Post**	***SEM***	**Challenge**	**Diet**	**Challenge x Diet**
n	5	5	6	6				
BW, kg[1]	38.9	44.3	37.0	40.6	*0.83*	-	-	-
N digestibility, %	94.7	94.9	92.4	93.1	*0.28*	0.14	0.001	0.27
N balance, g/(kg $BW^{0.75}$·d)								
N intake	2.32	2.32	1.77	1.75	*0.061*	0.17	< 0.001	0.23
Faecal N excretion	0.12	0.12	0.13	0.12	*0.003*	0.16	0.41	0.34
Urinary N excretion	0.64	0.73	0.42	0.43	*0.031*	0.04	< 0.001	0.14
N retention	1.57	1.47	1.22	1.20	*0.038*	0.07	< 0.001	0.28
N retention/ digestible N intake, %	71.2	66.9	74.6	73.3	*0.96*	0.07	0.02	0.29

[1]Average body weight (BW) based on measurements at 1 d before the start of each N balance period and at the last d of the N balance period.

Table 5.3 Effect of CFA challenge and dietary protein supply (Adequate, A or Restricted, R) on the irreversible loss rate (ILR, µmol/(kg BW·h)), release from protein breakdown (µmol/(kg BW·h)), and pool size (µmol/kg BW) of plasma amino acids (AA) in growing pigs (mean values with the pooled SEM).

Diet	**Adequate (A)**			**Restricted (R)**				***P*-value**		
Day (pre- or post-challenge)	**Pre d -5**	**Post d 3**	**Post d 8**	**Pre d -5**	**Post d 3**	**Post d 8**	***SEM***	**Day**	**Diet**	**Day · Diet[2]**
Lys, n	5	5	5	4	6	6				
ILR	695	716	698	559	605	576	*18.2*	0.61	0.02	0.95
Breakdown[1]	625	648	631	510	556	528	*17.2*	0.60	0.03	0.96
Pool size	85	79	108	90	74	53	*6.6*	0.88	0.08	0.23
Trp, n	4	4	-	4	6	1				
ILR	83	77	-	87	70	63	*3.2*	0.27	0.80	0.49
Breakdown	71	65	-	78	62	55	*3.2*	0.28	0.69	0.48
Pool size	16	16	-	22	14	27	*1.4*	0.13	0.39	0.27
Met, n	5	5	4	4	6	4				
ILR	271	267	295	221	237	198	*10.1*	0.91	0.03	0.21
Breakdown	235	232	261	196	212	174	*9.6*	0.91	0.06	0.21
Pool size	42	27[a]	88[b]	36	39	24	*6.4*	0.24	0.16	0.03
Ile, n	5	5	5	5	6	6				
ILR	450	464	426	400	400	379	*9.9*	0.24	0.05	0.86
Breakdown	374	390	354	346	347	328	*9.0*	0.29	0.21	0.85
Pool size	85	73	78	84	75	55	*5.8*	0.57	0.46	0.73
Leu, n	5	5	5	5	6	6				
ILR	797	744	732	690	612	633	*25.9*	0.33	0.13	0.92
Breakdown	658	610	601	592	516	539	*24.5*	0.37	0.30	0.92
Pool size	136	89	106	118	92	87	*9.6*	0.32	0.57	0.88
Val, n	5	5	5	5	6	6				
ILR	632	655	600	548	550	520	*11.9*	0.04	< 0.01	0.74
Breakdown	536	562	509	481	484	455	*10.2*	0.10	0.02	0.73
Pool size	150	135	138	142	128	93	*9.1*	0.49	0.19	0.70
Phe, n	5	5	5	5	5	6				
ILR	318	312	309	269	266	268	*8.8*	0.87	0.09	0.99
Breakdown	250	246	245	220	219	221	*8.0*	0.94	0.30	1.00
Pool size	59	57	63	57	58	60	*4.5*	0.94	0.84	0.98
Tyr, n	5	5	5	5	6	6				
ILR	347	340	312	290	280	270	*7.4*	0.06	0.01	0.71
Breakdown	295	290	263	253	244	235	*6.5*	0.09	0.03	0.73
Pool size	86	72	78	79	79	79	*5.7*	0.88	0.99	0.91

[1]AA released from protein breakdown was calculated as the difference between ILR and intake, using the steady state model of Waterlow (2006), *i.e.* ILR = protein breakdown + dietary intake = protein synthesis + AA oxidation. Intake was estimated by multiplying the feed intake by the dietary AID content of each AA and dividing by 24 h and the molar mass.
[2]In case of significant interactions, means without a common letter differ.

ILR for Val was affected by day of collection (P = 0.04), it remained constant until 3 day post-challenge, but was 7% lower (P = 0.05) at day 8 post-challenge (Table 5.3). ILR for Tyr tended to be affected by day of collection (P = 0.06), with a 9% lower (P = 0.06) ILR at day 8 post-challenge than at day -5 pre-challenge. Val release in plasma from protein breakdown tended to be affected by day of collection (P = 0.10), with a 6% lower (P = 0.09) breakdown rate at day 8 post-challenge than at day 3 post-challenge. Tyr release from protein breakdown tended to be affected by day of collection (P = 0.09), with a 10% lower (P = 0.09) breakdown rate at day 8 post-challenge than pre-challenge. The pool size of Lys, Trp, Ile, Leu, Val, Phe and Tyr was not affected by day of collection. Met pool size was approximately 230% greater at day 8 than at day 3 post-challenge in A-pigs, but not in R-pigs (P = 0.03) (Table 5.3).

Correlations ILR vs. *NAPI, HAPI and N retention*

Results of the correlation analyses between ILR or ILR index of AA and NAPI, HAPI, or N retention are presented in Table 5.4. The ILR index was not affected by dietary protein supply. Positive correlations were observed between ILR and N retention for all AA, except for Trp. ILR of the sum of all measured AA was positively correlated with N retention. ILR index did not correlate with N retention for any of the AA measured.

Table 5.4 Correlation coefficients for the relationships between irreversible loss rate (ILR) index and nutritional acute phase index (NAPI), for ILR index and health status acute phase index (HAPI), and for ILR and N retention. Correlations were calculated for data obtained at day 3 post-challenge and pre-challenge, and for data obtained at day 8 post-challenge and pre-challenge in growing pigs.

	Pre- and day 3 post-challenge	**Pre- and day 8 post-challenge**
ILR index for Trp *vs*. NAPI	-0.45; P = 0.06[1]	NS
ILR index for Val *vs*. NAPI	NS	-0.38; P = 0.09
ILR index for Tyr *vs*. NAPI	NS	-0.48; P = 0.03
ILR index for Trp *vs*. HAPI	-0.41; P = 0.09	NS
ILR index for Val *vs*. HAPI	NS	-0.38; P = 0.09
ILR index for Tyr *vs*. HAPI	NS	-0.51; P = 0.02
ILR for Lys *vs*. N retention	0.49; P = 0.03	0.57; P = 0.01
ILR for Met *vs*. N retention	0.47; P = 0.04	0.55; P = 0.02
ILR for Ile *vs*. N retention	0.50; P = 0.02	0.53; P = 0.01
ILR for Leu *vs*. N retention	0.36; P = 0.10	0.51; P = 0.02
ILR for Val *vs*. N retention	0.64; P < 0.01	0.65; P < 0.01
ILR for Phe *vs*. N retention	0.50; P = 0.02	0.50; P = 0.02
ILR for Tyr *vs*. N retention	0.65; P < 0.01	0.68; P < 0.01
Sum of ILR *vs*. N retention	0.58; P < 0.01	0.69; P < 0.001

[1]Pearson correlation coefficients are presented with their P-value. Each correlation analysis included data of 11 pigs.

Discussion

The aim of the present study was to quantify the effect of immune system activation on N retention and AA metabolism in growing pigs, depending on dietary protein supply. Intravenous CFA administration has been previously used in pigs as a model for immune system activation (Edwards and Slauson, 1983; Melchior *et al.*, 2004; Melchior *et al.*, 2005). In the present study, *i.v.* CFA administration activated the innate immune system, as indicated by a 2 to 4 fold increase in serum concentrations of CRP, haptoglobin, and pigMAP. The observed increase in haptoglobin concentration is in accordance with a study of Melchior *et al.* (2005) in CFA-challenged pigs. Similarly, haptoglobin and pig-MAP concentrations increase up to 6 fold and CRP concentrations up to 4 fold in response to bacterial and parasitic infections, or inflammation induced by *s.c.* turpentine challenge in pigs (Heegaard *et al.*, 2011). The observed reduction in WBC at day 1 post-challenge might be due to leukocyte migration into the lungs, as infiltration of neutrophils and eosinophils has been reported in lung tissue after *i.v.* CFA administration in pigs (Edwards and Slauson, 1983). Autopsy results in the present study also indicate increased infiltration of lymphocytes and macrophages in lung and liver following CFA challenge. The drop in WBC at day 0 pre-challenge was, however, unexpected.

In the present study, dietary protein supply did not affect relative lung weight of the pigs following CFA administration. In contrast, Le Floc'h *et al.* (2008) observed greater lung weight in CFA-challenged pigs fed a Trp deficient diet than in pigs fed an adequate Trp diet. In the present study, the restricted dietary protein supply reduced pre-challenge serum CRP concentrations and tended to reduce serum albumin concentrations pre- and post-challenge. In line with our results, plasma albumin concentrations, and albumin fractional and absolute synthesis rate decreased in pigs (Jahoor *et al.*, 1999) fed low protein diets and after *s.c.* turpentine challenge. In addition, a lower plasma albumin concentration and albumin fractional synthesis rate was observed in *i.m.* LPS challenged pigs fed a low Met + Cys diet than in pigs fed a diet with an adequate Met + Cys content (Litvak *et al.*, 2013a). These findings may suggest that the dietary AA supply can be insufficient for albumin synthesis, independent of immune system activation. Albumin serves as a nutrient carrier and depot by binding to nutrients (Cray, 2012; Fanali *et al.*, 2012). This carrying capacity is possibly reduced when dietary protein supply is restricted. Houdijk *et al.* (2007) found a reduction in plasma CRP concentrations when the dietary protein content decreased in pigs with sub-clinical colibacillosis. Our results indicate that serum CRP concentrations are sensitive to dietary protein supply in the absence of immune system activation. Upon immune system activation, however, serum CRP concentrations were unaffected by dietary protein supply. This is in line with the concept that, immune functions are prioritised over other body functions during immune system activation (Klasing and Johnstone, 1991; Lochmiller and Deerenberg, 2000). The tendency for lower serum albumin concentrations during the pre- and post-challenge period in R-pigs than in A-

pigs, however, indicates that a restriction in dietary protein supply can reduce albumin concentrations independent of immune system activation in growing pigs. Furthermore, this suggest that, in contrast to prioritizing for immune functions, in this case APP synthesis, there is a competition for AA between immune functions and other body functions such as body protein deposition in growing pigs.

The ILR of an AA reflects the amount of free AA that disappears per unit of time from the blood plasma pool. ILR includes use of AA for protein synthesis and oxidation, and does not distinguish between both fluxes. The ILR of AA in plasma in combination with the pool size or concentration of AA is, however, more useful for quantifying changes in AA metabolism than merely plasma AA concentrations or pool sizes. Changes in AA metabolism, *e.g.* an increased protein synthesis rate, can occur without concomitant changes in plasma AA concentrations or pool size, as AA concentrations can be maintained when fluxes from protein intake, breakdown and synthesis of body protein, and oxidation of AA are changing (Waterlow, 2006). Yet, changes in plasma AA concentrations have been used previously as a measure to asses effects of immune system activation on AA metabolism (Maes *et al.*, 1993; Melchior *et al.*, 2004; Melchior *et al.*, 2005; Le Floc'h *et al.*, 2006). In the present study, the lower ILR for Val at day 8 than day 3 post-challenge was not associated with a change in pool size. Therefore, the use of pool size or AA plasma concentrations as a single measure to quantify effects of immune system activation on AA metabolism can be misleading. Furthermore, N retention reflects the total whole body protein deposition, and does not distinguish between N retained in muscle protein or APP, neither does the ILR.

The restricted dietary protein supply reduced apparent faecal N digestibility compared to the adequate dietary protein supply. This is likely attributed to a proportionally greater excretion of basal endogenous N in R-pigs, as the relative contribution of endogenous N to total faecal N excretion decreases with increasing dietary protein supply (Fan *et al.*, 1994) or when AA are administered *i.v.* (de Lange *et al.*, 1989). N retention was 20% lower in R-pigs than in A-pigs, and corresponded with the observed reduction in ILR for the presented AA, except for Trp. ILR for Lys was 19% lower in R-pigs, followed by Met (-18%), Leu (-16%), Phe (-15%), Val (-14%), and Ile (-13%). In addition, positive correlations were observed between N retention and ILR for Lys, Met, Ile, Leu, Val, Phe and Tyr, but not for Trp. These positive correlations were mostly attributed to differences in dietary protein supply, as the ILR indices for all AA, reflecting the changes in ILR within animals due to the challenge, did not correlate with N retention. As expected, the restricted dietary protein supply resulted in a lower urinary N excretion (absolute in g/kg $BW^{0.75}$/day as well as relative as % of N intake), indicating that oxidation of AA was reduced in R-pigs compared to A-pigs.

In the present study, immune system activation induced by CFA altered N retention and AA metabolism, independent of dietary protein supply. It was hypothesized that the effect of CFA challenge on protein metabolism would be more pronounced under conditions of a marginal dietary protein supply. This would increase the competition for indispensable AA used for immune system functioning and for body protein deposition in muscle as a main determinant of the animal's growth. The effects of CFA

challenge on variables related to protein metabolism, however, were less pronounced in R-pigs than in A-pigs. In R-pigs there was no drop in N retention post-challenge compared to pre-challenge, suggesting that there is a high priority for the allocation of AA for body protein deposition in R-pigs. N utilisation for retention, *i.e.* N retention / digestible N intake, was greater in R-pigs than in A-pigs as expected due to the difference in dietary protein supply. As shown by Fuller *et al.* (1987), the increase in N retention associated with an increase in dietary protein supply, is proportionally smaller than the increase in N digestibility. In A-pigs, N retention numerically decreased by 6% post-challenge and the post-challenge drop in ILR for Val and Tyr is therefore most likely attributed to a reduction in protein synthesis. In contrast, N retention in R-pigs was unaffected by CFA challenge. Therefore, the post-challenge drop in ILR for Val and Tyr in R-pigs, can probably be attributed to a reduction in oxidation rather than to a reduction in body protein synthesis, as also indicated by the concomitant numerical decrease in Val pool size. This indicates that immune system activation reduced Val and Tyr oxidation in R-pigs, but not in A-pigs. In humans, Leu oxidation decreased substantially more than Leu utilisation for protein synthesis, with 77% and 30% respectively, when a low compared to a high protein diet was provided (Hoerr *et al.*, 1993). A decrease in AA oxidation is possibly a compensatory mechanism in R-pigs to spare AA from catabolism, when AA for protein synthesis are scarce. CFA challenge increased urinary N excretion, and tended to reduce N retention and N utilisation for retention. In line, greater urinary N excretion (de Ridder *et al.*, 2012) and lower N retention (Williams *et al.*, 1997; de Ridder *et al.*, 2012) was observed in pigs with an activated immune system by continuous exposure to major vectors of antigen transmission (Williams *et al.*, 1997) or by repeated *i.m.* LPS injections (de Ridder *et al.*, 2012). The observed greater urinary N loss in the post-challenge period might be caused by increased AA oxidation of unbalanced AA, as suggested by Reeds *et al.* (1994). An increase in the synthesis of APP is suggested to increase the demands for AA, especially Phe, Trp, and Tyr, which can be released by breakdown of muscle protein (Reeds *et al.*, 1994). As the AA composition of muscle protein differs from that of APP, an imbalance in AA available for body protein synthesis can occur, leading to greater urinary N losses (Reeds *et al.*, 1994). It was hypothesized that an increase in serum APP during immune system activation affects the ILR of AA, with concomitant reduction in pool size, due to increased incorporation of (in particular aromatic) AA into APP, and increased pool size and oxidation of non-limiting AA resulting from related AA imbalance. The 2 to 4 fold increase in APP concentrations following immune system activation was, however, not associated with an increase in ILR of any of the AA. In contrast, the ILR for Val was lower at day 8 than at day 3 post-challenge and CFA challenge tended to reduce the ILR for Tyr at day 8 post-challenge compared to day -5 pre-challenge. In addition, negative correlations were observed between NAPI or HAPI and the ILR index for Tyr, and tendencies for negative correlations with the ILR index for Trp and Val. These findings could on the one hand suggest that the changes in AA utilisation for growth due to the incorporation into APP are quantitatively less important than expected based on findings in other studies (Reeds *et al.*, 1994; Iseri and Klasing, 2013). On the other hand, and more likely, a decrease in muscle protein

synthesis during immune system activation (Breuille, 1994) might have balanced the increase in AA utilisation after immune system activation due to increased incorporation of AA into APP. In the present study, however, no distinction could be made between AA utilisation for APP synthesis or for muscle protein synthesis related to growth.

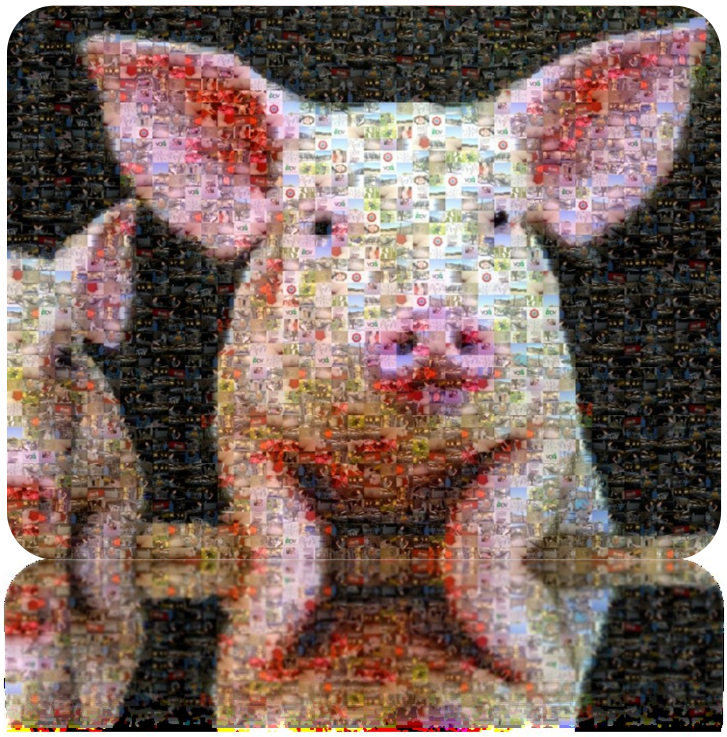
Another explanation for the lower ILR for Tyr at day 8 post-challenge may be a reduced formation of Tyr from Phe. Phenylalanine-hydroxylase catalyses the formation of Tyr from Phe. Pro-inflammatory cytokines (*e.g.* Interferon-γ), induce expression of the guanosine-triphosphate-cyclohydrolase-1 enzyme pathway and concomitantly release reactive oxygen species, which in turn inhibit phenylalanine-hydroxylase activity (Capuron *et al.*, 2011). Thus, the formation of Tyr could be reduced after CFA challenge.

Met pool size was approximately 230% greater at day 8 than at day 3 post-challenge in A-pigs, but not in R-pigs (P = 0.03). The greater pool size at day 8 in A-pigs might be attributed to greater release of Met from protein breakdown, *i.e.* Met released from breakdown increased by 11% compared to pre-challenge, and by 13% compared to day 3 post-challenge. An increase in plasma pool size at similar ILR may indicate Met oxidation (transsulfuration) rather than utilisation for protein synthesis. Hence, Met may have been released in excess of its requirement, which corresponds with the relatively high Met content in muscle protein (Conde-Aguilera *et al.*, 2010) compared with average APP (Reeds *et al.*, 1994). It can be expected that Met is increasingly used for conversion into Cys in order to produce glutathione, which plays an important role in maintaining antioxidant defences (Grimble and Grimble, 1998; Malmezat *et al.*, 2000), and supports proliferation of T lymphocytes (Grimble, 2006). As shown by Litvak *et al.* (2013b) immune system activation by *i.m.* LPS administration increased the optimal dietary Met to Met + Cys ratio for whole body protein deposition.

In conclusion, the effect of CFA challenge on N retention and AA metabolism was largely independent of the dietary protein supply. A deficient dietary protein supply decreased blood serum concentrations of CRP and to a lesser extent albumin, stressing the importance of an adequate dietary AA supply for the production of APP in growing pigs. Immune system activation via *i.v.* CFA administration increased urinary N excretion in growing pigs, and tended to reduce N retention and N utilisation of digestible N for retention. Immune system activation reduced the ILR for Val and Tyr, but did not lead to a significant change in pool size of the measured AA, except for Met. ILR of all AA measured, except for Trp, were strongly affected by dietary protein supply and were positively correlated to N retention. Correlations between ILR and APP indices were absent or negative, indicating that changes in AA utilisation for APP synthesis are quantitatively unimportant in growing pigs, or, more likely, outweighed by a decrease in muscle protein synthesis during immune system activation.

Chapter 6

General Discussion



Introduction

The main objective of the present thesis was to quantify the effect of health status on amino acid (AA) requirements for body protein deposition and for immune system functioning of growing pigs. In the present Chapter, the findings of four studies are discussed, with emphasis on the health status of farms and experiments were the immune system is activated under more standardized conditions, the techniques employed to estimate AA requirements, and the nutritional costs related to the functioning of the immune system (Figure 6.1). Finally, an overview of the main conclusions of this thesis and recommendations for the optimization of diets of growing-finishing pig farms differing in their health status are presented.

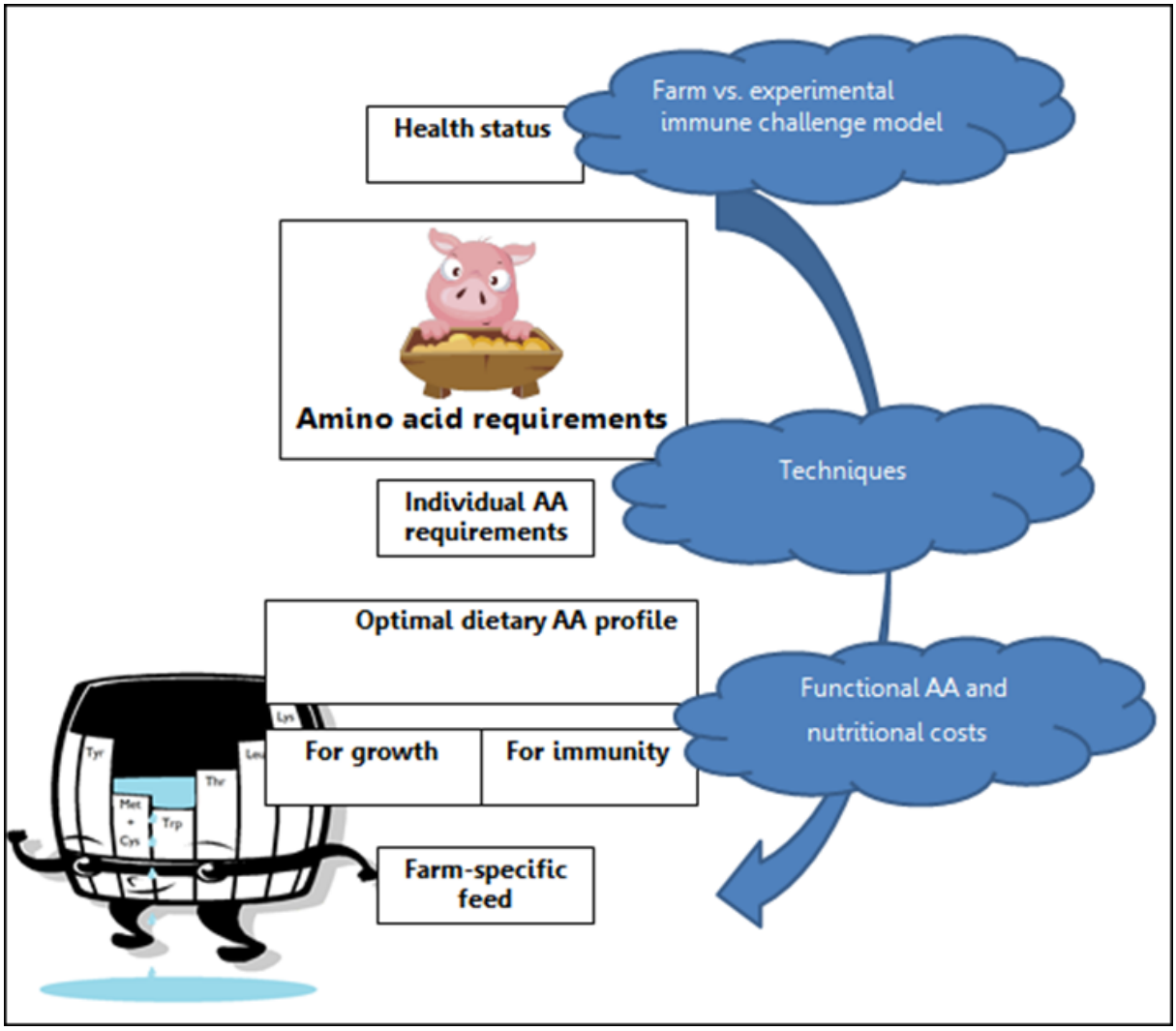


Figure 6.1 Schematic presentation of the outline of the General Discussion.

Health status

Variation in health status between pig farms vs. experimental models

For the implementation of targeted feeding strategies for particular groups of farms sharing a common health status, it is pivotal to classify farms on the basis of their health status. In the study described in Chapter 2, a health status web was developed as a concept for the classification of growing-finishing pig farms, based on data recorded in current commercial practice. This concept incorporates traits which are recorded at farm level, are readily available and are collectively related to the incidence of (sub)clinical disease. The data on the six traits of farms incorporated in the health status web were collected over a period of one year. Thus, this concept reflects the long term health status of a farm, rather than a health status that is influenced by short term incidents. The health status web aims to provide insight into the variation in health status between farms.

(Sub)clinical infections that often occur in the commercial growing-finishing pig industry include *Ascaris suum*, Porcine circovirus type 2 (PCV2), *Streptococcus suis*, Porcine reproductive and respiratory syndrome virus (PRRSV), *Lawsonia*, *Pasteurella multocida*, and *Bordetella bronchiseptica* (Figure 1.4; General introduction). In addition, behavioural abnormalities and health related problems such as increased incidence of tail or ear biting and leg problems have been associated with elevated concentrations of acute phase proteins (APP) in blood (Petersen *et al.*, 2002; Salamano *et al.*, 2008; Piñeiro *et al.*, 2013). All health challenging conditions observed in commercial growing-finishing pig operations share one common aspect, *i.e.* they induce immune system activation and affect protein and energy metabolism (Figure 1.8; General ntroduction). The traits included in the health status web are collectively related to (sub)clinical disease, which in turn induce immune system activation. Experimental models are, however, required to quantify the effect of health status on AA requirements for body protein deposition and for immune system functioning of growing pigs. Pastorelli *et al.* (2012) performed a meta-analysis on data of 122 challenge studies to quantify the effect of experimental challenge models that activate the immune system on average daily gain (ADG), feed intake and feed efficiency (Figure 6.2). According to their findings, especially bacterial infections of the gastro-intestinal tract greatly reduced ADG, by 40% on average relative to unchallenged control, followed by mycotoxicoses and respiratory diseases. Depending on the type of immune system challenge, less than 30% to more than 70% of the reduction in ADG was due to a reduction in feed intake, the first in case of bacterial infections of the gastro-intestinal tract and poor housing conditions, and the latter in case of lipopolysaccharide (LPS) challenge, mycotoxicoses and respiratory diseases (Pastorelli *et al.*, 2012).

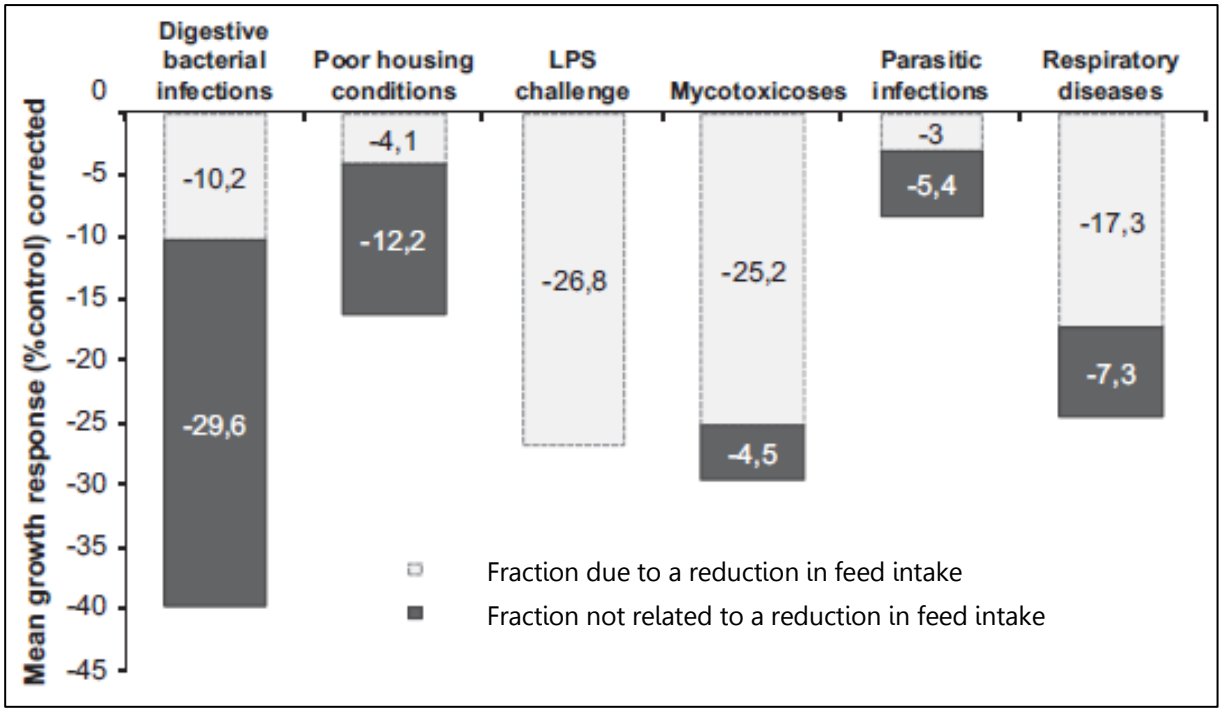


Figure 6.2 Partitioning of the reduction in the average daily gain (ADG) following an immune challenge between the fraction due to a reduction in feed intake (*i.e.* the extent of change in ADG associated with a reduction in feed intake) or the fraction not associated with a reduction in feed intake, but related to a greater maintenance requirement; the change in ADG at similar feed intake) in pigs (adapted from Pastorelli *et al.*, 2012).

To create a standardized contrast in immune system activation an intravenous (*i.v.*) CFA-challenge inducing a chronic, non-infectious lung inflammation was used as an experimental challenge model (Chapter 5). In addition, a contrast in health status between pigs (high *vs.* suboptimal) was created by selecting two farms at which pigs differed in the presence of antibodies in blood against a number of pathogens (Chapter 4). This contrast in health status was created in order to determine the effects of health status on nitrogen (N) retention and AA utilization in growing pigs, simulating what is observed in practice.

In search of an experimental model to quantify the effect of immune system activation on AA requirements for body protein deposition and for immune system functioning of growing pigs the following criteria were set to induce:

- a standardized response of the immune system, associated with an increase in body temperature and elevated concentrations of APP in blood, with a magnitude similar to that observed in pigs with an activated immune system due to *e.g.* (sub)clinical, over a time period of at least several days;
- a low variation in response between animals with regard to immunological parameters and changes in AA metabolism;
- a minimal effect on feed intake.

In addition, the discomfort imposed on the animal was also taken into account. A pilot study was conducted (Textbox I) to compare the use of *i.v.* injection of Complete Freund's Adjuvant (CFA), associated with a sterile lung inflammation, and *s.c.* injection with turpentine oil (TO), associated with tissue damage, to activate the immune system and to study the effect of immune system activation on AA metabolism in pigs. Although it is difficult to compare and judge the discomfort caused by the two challenge-models, CFA was preferred over TO. Considering the smaller within animal variation in response of APP and ILR in CFA challenged pigs compared to TO challenged pigs, and the greater magnitude of response in APP and ILR, CFA was chosen as the most appropriate experimental model to be used in Chapter 5, for the quantification of the effect of immune system activation on AA metabolism and N retention. The pigs from a farm characterized as having a low health status in the study described in Chapter 4 had greater leukocyte counts and serum haptoglobin concentrations, and coinciding lower serum albumin concentrations during the experiment. Yet, they were able to show compensatory growth under adequate dietary AA supply. Moreover, in the high health status pigs (Chapter 4), the observed serum APP concentrations were relatively high in comparison to concentrations observed in unchallenged pigs in Chapter 5, and in clinically healthy SPF pigs in the study of Parra *et al.* (2006), indicating that under commercial conditions, even greater contrasts in concentrations of APP are observed. The experiment in Chapter 4 indicates that it is very difficult to maintain a contrast in health status, in this case by housing pigs in unsanitized stables *vs.* desinfected stables in respiration chambers with high efficiency particle airfilters and applying contrasting hygienic and management measures.

Textbox I Challenge models to study the effect of immune system activation on amino acid metabolism in pigs (E. Kampman - van de Hoek, 2013).

Objective

The objective of the present study was to compare the use of Complete Freund's Adjuvant (CFA) and turpentine oil (TO) to activate the immune system and to study the effect of immune system activation on AA metabolism in pigs.

Material and Methods

Eight pigs (30 kg BW) were challenged with either four *i.v.* CFA infusions (n = 3)), a single *s.c.* TO injection (n = 3), or *i.v.* and *s.c.* saline as a control (control, n = 2). Restricted feed intake of 2.7 · the estimated ME requirements for maintenance. Feed intake was determined daily. Plasma acute phase protein (APP) concentrations were determined immediately before and at day 1, 2 and 6 after the challenge. Leukocyte counts were determined daily. A 4-d N balance was performed (day 0 to 3 after the challenge). At day 2, a mixture of 7 universally ^{13}C-labelled essential AA (Lys, Met, Trp, Ile, Leu, Val, Phe, Tyr) was infused *i.v.* as a bolus to study the irreversible loss rate (ILR). At 9 d after the start of the challenge, pigs were euthanized after which autopsy was performed.

Results

One day after the start of the challenge serum haptoglobin concentrations increased 9 fold (P = 0.03) in CFA compared to control pigs, but was not increased in TO pigs (Figure Ia). Serum CRP concentrations increased three fold in CFA pigs (P = 0.06) and in TO-pigs (P = 0.03) compared to control pigs. PigMAP increased 6 fold (P < 0.01) only in TO, and CRP increased 4 fold in TO (P = 0.01) compared to control. CFA increased (P < 0.05) eosinophil counts in blood at day 1 and 2. Feed intake was reduced at day 0 and 1 in CFA and at day 1 in TO challenged pigs. N retention was similar in pigs of each of the three groups.

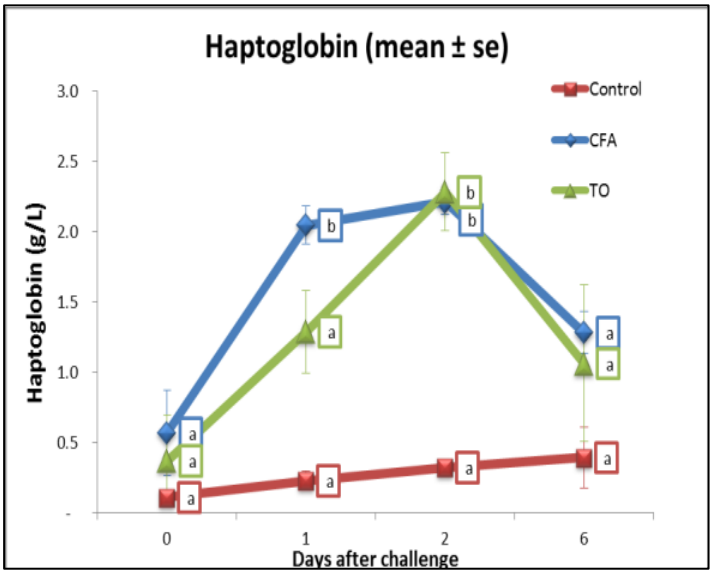


Figure Ia Serum haptoglobin concentration of pigs before and after immune system activation with CFA, TO or saline (control).

The ILR for Lys, Met, Trp, Ile, Leu, Val, Phe and Tyr were numerically lower in CFA pigs compared to control pigs, with the most pronounced numeric difference in ILR for Tyr, followed by Leu, Met, and Phe (Figure Ib). In TO pigs, the ILR were almost similar to the control pigs (Figure Ib).

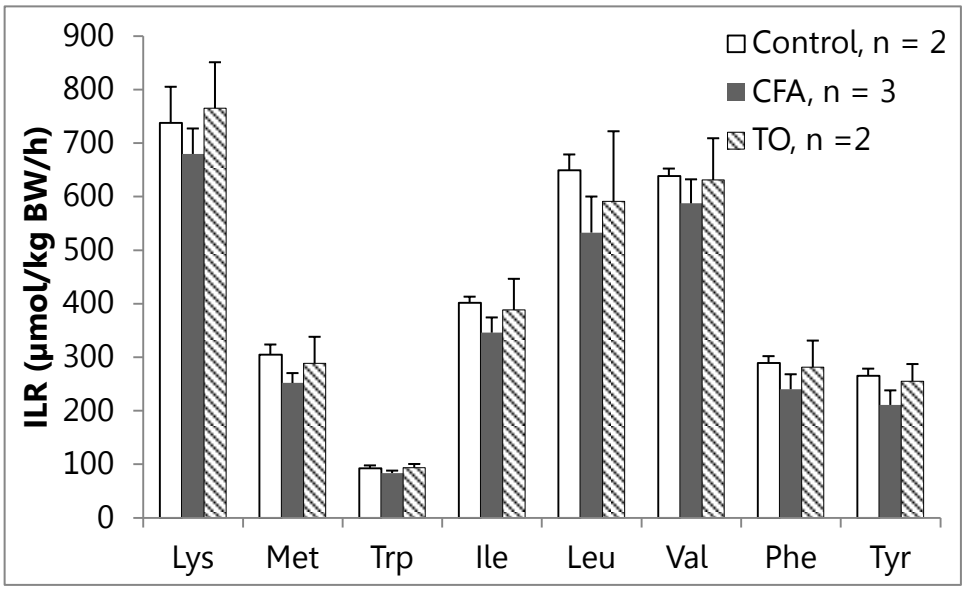


Figure Ib Effect of CFA or TO administration on the irreversible loss rate of plasma amino acids (AA) by intravenous bolus infusion of universally labelled ^{13}C-AA in growing pigs.

Conclusions

The reduction in ILR of AA in pigs with an activated immune system as induced by the CFA challenge indicates a reduction in protein synthesis and/or oxidation of AA. TO administration resulted in less pronounced differences in ILR. Possibly, this change in utilization is due to repartitioning of AA to protein synthesis for the immune system at the expense of synthesis for net body protein deposition.

Adaptation and flexibility

In Chapter 4, a large difference of approximately 6 kg in starting BW was observed between pigs of similar age deriving from farms characterized as having a low or high health status. Low health status pigs receiving a diet adequate in AA, showed higher ADG relative to BW and metabolic BW than high health status pigs, whereas a dietary deficiency of Met + Cys, Thr and Trp did not allow compensatory growth to occur in low health status pigs. Thus, low health status pigs receiving a diet adequate in AA were able to show compensatory growth related to a more efficient N utilization. In line, Kyriazakis and Emmans (1992) suggested that impairment in growth following a period of nutrient limitation can be corrected over time, depending on the availability of resources during rehabilitation. Compensatory growth or improved N retention has been previously demonstrated in pigs following a period of restricted feeding (Critser *et al.*, 1995; Bikker *et al.*, 1996a; Bikker *et al.*, 1996b) or restricted protein and AA supply (Tullis *et al.*, 1986; Fabian *et al.*, 2004). The compensatory growth or N retention has been attributed to increased feed intake (Critser *et al.*, 1995), improved feed conversion ratio when feed intake was kept similar between restricted and control pigs (Bikker *et al.*, 1996b), or due to reduced excretion of urinary N excretion (Fabian *et al.*, 2004). The meta-analysis study on feed intake and ADG responses of pigs to an immune challenge (Pastorelli *et al.,* 2012) showed that the ADG of challenged pigs was lower during a challenge, but in case of respiratory diseases, pigs were able to fully recover, *i.e.* have a similar ADG compared to control pigs. The authors suggested that compensatory growth or feed intake occurred during and after an immune system challenge, as no difference in feed intake was observed once challenged pigs were recovered. Pigs *i.m.* challenged with LPS were not able to compensate for the reduction in BW due to the challenge (Moraes *et al.*, 2012). In line, the findings in Chapter 4 suggest that in pigs with a low health status compensatory growth is possible following a period of reduced growth, especially when providing a diet adequate in AA, but not when providing a diet deficient in Met + Cys, Thr and Trp.

The compensatory growth observed in the studies of Bikker *et al.* (1996a; 1996b) has been suggested to occur primarily due to protein deposition in organs and via an increase in digesta weight in the small intestine, and is to a lesser extent related to an increase in carcass protein deposition. In the present thesis no measurements on compensatory effects in different body components were performed. Another example that shows that animals are flexible in coping with nutritional deficiencies is shown by the study of Conde-Aguilera *et al.* (2010), which suggests that animals can either reduce body protein deposition for growth or change the composition of growth. In that study a dietary sulphur AA deficiency reduced the Met and Cys concentration of different body proteins, especially in skeletal muscle. Nevertheless, this adaptation mechanism may be limited as a reduction in ADG was not prevented by the observed reduction in AA content of body protein tissues (Conde-Aguilera *et al.*, 2010), and suggests that animals may not be very flexible in changing the composition of body muscle protein. In PRRSV challenged pigs whole body concentrations of dry matter and lipid decreased, whereas concentrations of protein and ash increased compared to

non-challenged control pigs (Escobar *et al.*, 2004). Furthermore, from day 7 to 14 post inoculation with PRRSV, the composition of gain consisted of protein and ash but not lipid, as the amount of lipid did not change during this period in PRRSV challenged pigs.

Techniques to estimate AA requirements

In the present thesis, two techniques were evaluated. A simplified dose-response technique to quantitatively estimate a change in AA requirements of individual meal-fed pigs (Chapter 3), and an isotope dilution technique, to quantify the effect of immune system activation on AA metabolism and urea production (Chapter 4 and 5). The dose-response technique with a decreasing Lys supply in time and a step length of 3 day with urinary N excretion as response criteria proved to be a simple, accurate technique to quantitatively estimate a change in AA requirements of individual meal-fed pigs. Nevertheless, a minimum time period of 21 days is required for each individual (a step length of 3 days · at least 7 dietary AA levels). An experimental model that maintains a standardized extent of immune system activation with systemic effects that are believed to influence AA metabolism for a period of at least 21 days was not found in literature. Therefore, in the studies described in Chapter 4 and 5, the isotope dilution technique was used to quantify the effect of health status on AA requirements for body protein deposition and for immune system functioning of growing pigs, using experimental models to activate the immune system as described in Chapter 4 and 5. With minor changes the simplified dose-response technique may be further developed to determine the AA requirement of pigs from commercial farms with a different health status, using an oral dose of isotopic labelled urea and determine the change in urea enrichment in saliva as a response parameter for urea production. Furthermore, the level of immune system activation can be monitored by determining APP concentrations in saliva, as evaluated by Gutiérrez *et al.* (2009b). Taking saliva samples is far less invasive than taking a blood sample in pigs.

A schematic representation of the AA fluxes in the plasma pool, *i.e.* AA absorption from the diet and AA release from body protein breakdown, and AA fluxes out of the AA plasma pool, *i.e.* AA use for protein synthesis and AA oxidation, is provided in Figure 1.2 of Chapter 1. With the isotope dilution technique, the pool size and ILR, *i.e.* the amount of free AA that disappear per unit of time from the plasma pool for protein synthesis or oxidation, can be determined. The ILR is determined by measuring the change in plasma isotopic enrichment of individual AA in time after an *i.v.* administered bolus of U-^{13}C-labelled AA, while feeding the pigs hourly portions (Chapter 4 and 5). Under the assumption that there is a physiological steady state during the measurement of ILR and a constant size of the plasma pool, the turnover of AA in plasma (Q) equals:

AA absorption from the diet (I) + AA release from body protein breakdown (B) = AA use for protein synthesis (S) + AA oxidation (O).

ILR = S + O, B = Q - I = ILR - I.

The pool size and AA absorption flux are relatively small in relation to the breakdown and ILR flux, implying that that the AA pools are renewed 4 to 8 times per h, according to the data in Figure 6.3.

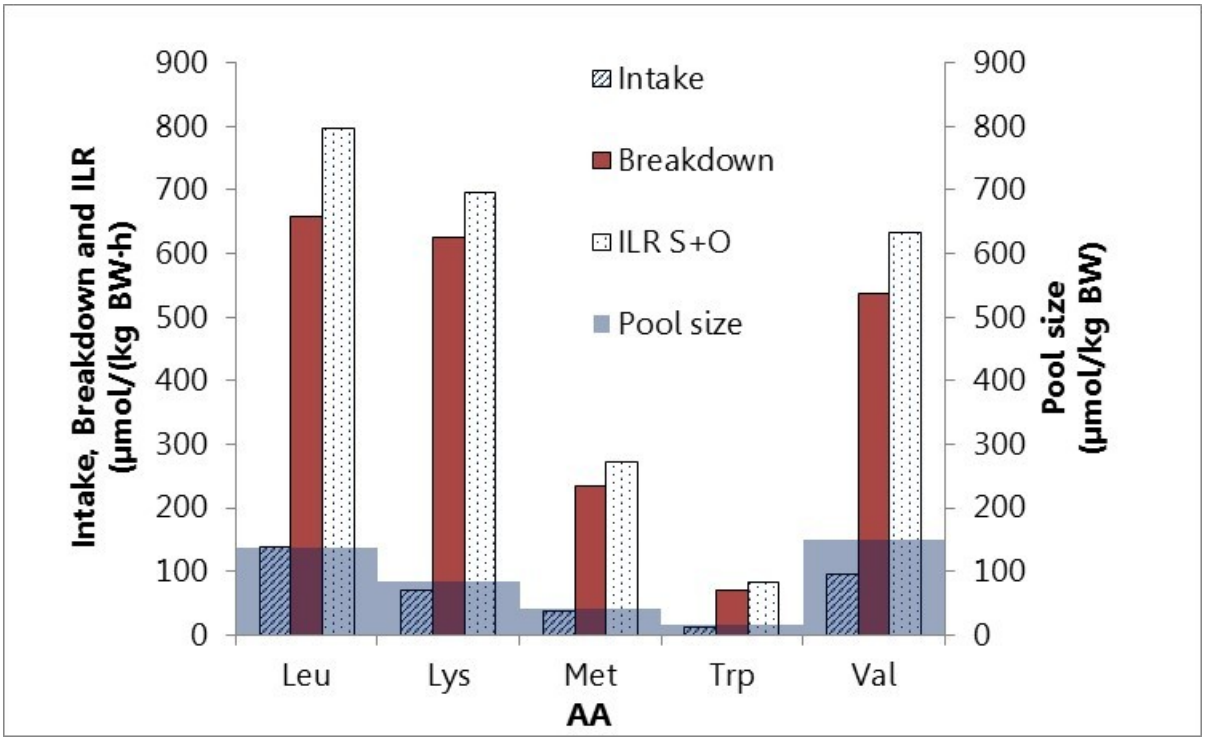


Figure 6.3 Mean of AA fluxes relative to pool size of clinically healthy pigs of approximately 42 kg body weight (BW) fed a diet adequate in protein and AA (Chapter 5).

An interesting feature of the isotope dilution technique is that it allows the simultaneous measure of the rate of utilization of various AA. The downside, as extensively discussed (Chapters 4 and 5) is that discrimination between synthesis and oxidation of each AA is not possible with this technique. Additional measurements can be performed to aid the interpretation of treatment effects on ILR, as was done in the studies of Chapter 4 and 5. These extra measurements include determination of AA pool size, rate of N retention, urea entry rate, urea pool size, and ^{13}C enrichment in plasma proteins. An overview of additional measurements done and how these can help in the interpretation of treatment effects on ILR for AA is presented below (Table 6.1).

To this end, three cases were selected from studies conducted in this thesis.

In **case 1** similar pool sizes were observed, although ILR and breakdown were different. Pigs fed a deficient protein and AA diet in Chapter 5, had a similar pool size of Met, Ile, Val, Phe and Tyr, while ILR and breakdown were lower, in line with a lower N retention (case 1a), compared to pigs fed an adequate protein and AA diet (case 1b).

This case shows the importance of measuring fluxes rather than only pool size or plasma concentrations.

In **case 2** the Met plasma pool size was greater at day 8 post-challenge compared to day 3, while the ILR was only slightly higher (Chapter 5). This likely reflects a higher rate of oxidation at the expense of utilization for protein synthesis. This would require measurements on urea entry rate and urea pool size to verify this assumption.

In **case 3**, especially in low health status pigs a numerical reduction in Trp pool size was observed, with a tendency for a lower ILR in pigs fed a diet deficient in Trp, compared to pigs fed a diet adequate in AA (Chapter 4). In low health status pigs fed the deficient diet, this coincided with a reduction in N retention, and a higher urea pool size reflecting that Trp is limiting (case 3a), while other AA became excessive and were oxidized (case 3b).

Case	AA ILR (S+O)	AA Pool size	Release from break-down (B)	Protein deposi-tion (S - B)	Urea entry rate	Urea pool size	Enrich-ment in plasma proteins
1a Deficient protein and AA diet	--	=	--	--			
1b Adequate protein and AA diet	++	=	++	++			
2 Increased oxidation	+	++	+++		x	x	
3a Limiting AA	--	--	= or +	--		++	
3b AA imbalance	++	--	= or + or -	--	++	++	==

Table 6.1 Physiological processes involved in three cases, selected from various chapters in this thesis. Changes in the observed physiological processes between two groups of pigs are reflected by ++ symbols indicating a greater response, by -- symbols indicating a lower response, and by == indicating a similar response. The x symbols indicate that measurements on that particular physiological process are required to verify the statement, while grey cells indicate that the measurements are not required.

Nutritional costs related to immune system activation

Summarizing the energetic costs associated with immune system activation, Lochmiller and Deerenberg (2000) found that the resting metabolic rate can increase with 1.1 to 1.6 fold due to infection, vaccination or endotoxin challenge as compared to controls

in human, sheep and rodents. As reviewed by Sandberg *et al.* (2007), the relative increase in energy expenditure in immune challenged animals due to antibody production ranged between 1.09 and 1.55 fold of control.

In the present thesis the focus is on the protein and AA costs related to immune system activation, divided into costs related to anabolic processes, especially an increase in synthesis of proteins for the immune system and repair of damaged tissues, and other associated metabolic costs. In order to quantify the nutritional cost related to immune system activation, the costs associated with the innate and acquired immune system need to be dissected. Furthermore, Isery and Klasing (2013) suggested that the division between early and late responses allows reallocation of nutrients to minimize nutritional costs. The magnitude of the immune response, *e.g.* acute or chronic immune system activation also affects the nutritional costs.

Innate and acquired components of the immune system

Efforts have been made to dissect the nutritional cost associated with the activation of different effector arms of the immune response, by quantifying the changes in mass of *e.g.* body tissues, like skeletal muscle and liver, cells and proteins, *e.g.* cytokines and APP (Houdijk *et al.*, 2001; Klasing, 2007; Iseri and Klasing, 2013). Results of different approaches in chickens indicate that a healthy young broiler chicken utilizes approximately 1.2% of the Lys intake for leukocyte production, antibody secretion and production of proteins (Klasing and Calvert, 1999; Klasing, 2007). In the healthy chicken, the main estimated Lys costs were related to immunoglobulin synthesis (59% of the total Lys costs for the immune system), leukocyte production (41%), and none to APP synthesis (0%). Following an LPS-challenge, however, anabolic processes, including mainly the hepatic acute phase response, increase the Lys use by the immune system to 6.7% of Lys intake, with 71% of the total Lys costs related to APP synthesis, 17% for leukocytes production and 13% for immunoglobulin synthesis (Klasing and Calvert, 1999). Further findings also indicate that the APP response is nutritionally more costly than the acquired immune response following an *E. coli* challenge in chickens (Iseri and Klasing, 2013), indicated by the sum of the weight of antibodies and APP, and leukocyte subpopulations pre- and post-challenge. The total weight of leukocytes was 676 mg/kg BW pre-challenge and increased with 54% at day 1 post-challenge, while the weight of APP was 333 mg/kg BW pre-challenge and increased with 207% at day 1 post-challenge (Figure 6.4). No estimates of nutritional costs related to the activation of the innate and adaptive immune system could be found in pigs. Although the sum of the mass of cells and proteins is not a direct measure for the amount of nutrients that are needed, the above mentioned studies show that the costs of an APP response is more substantial than that of an increase in leukocyte production and immunoglobulins synthesis.

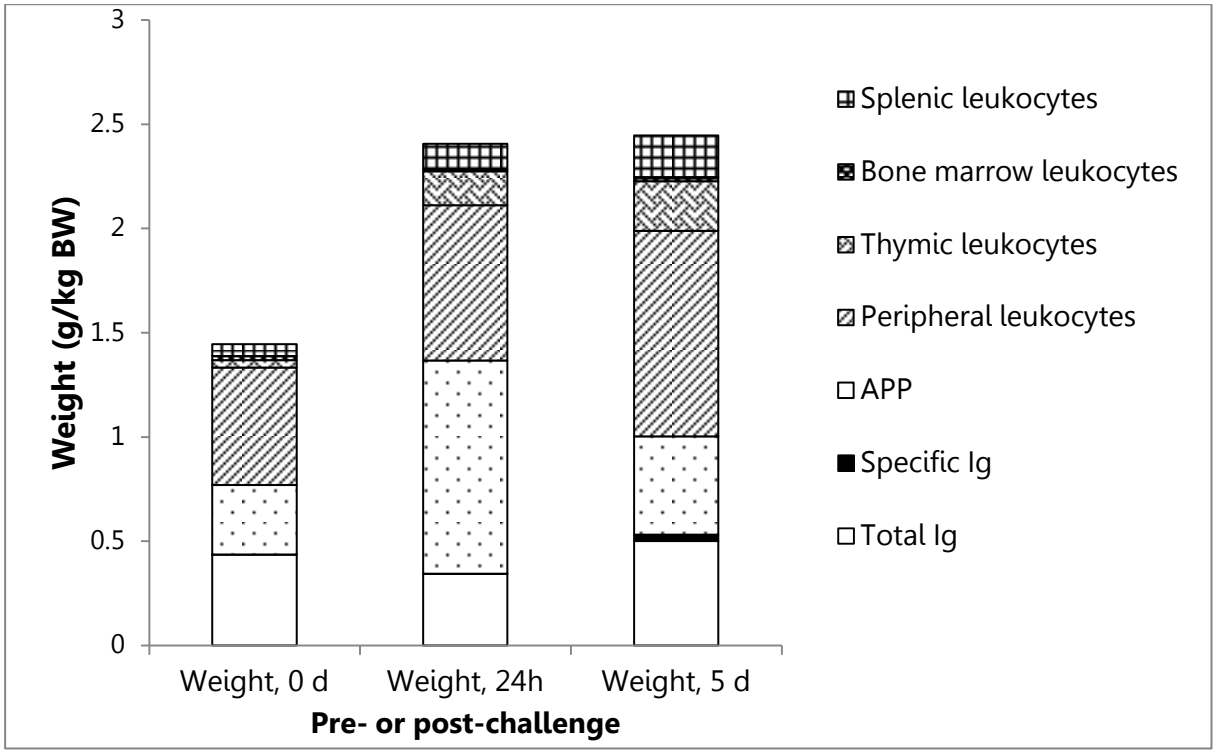


Figure 6.4 Weight of cellular and effector protein components of the systemic immune system of 1.8 kg chickens prior to and 24 h or 5 day following *E. coli* (*i.v.*) challenge (Iseri and Klasing, 2013).

Quantification of the nutritional costs of immune system activation

Quantifying the effect of immune system activation on AA requirements is important for future optimization of pig diets for farms with a specific health status. In this paragraph calculations on the estimated change in quantitative AA requirements for APP synthesis during an APP response in pigs after immune system activation are discussed. In addition, estimated changes in the optimal dietary AA profile after immune system activation are further evaluated based on the observed changes in AA metabolism in pigs with a different health status (Chapter 4) and in pigs with an experimentally induced chronic lung inflammation (Chapter 5).

Estimated costs of APP syntheses

Reeds *et al.* (1994) suggest that stimulation of the immune response in humans leads to an increased utilization of certain AA, particularly Phe, Trp and Tyr, for APP synthesis. However, their calculations were based on a typical APP response in humans after uncomplicated surgery, and on the AA composition of human APP, and the mean composition of bovine, porcine and ovine skeletal muscle. In this Chapter, similar calculations were performed for pigs (Textbox II).

The estimated APP synthesis (calculated as the rate of synthesis needed to sustain a serum concentration difference, assuming a particular half-life, specific for each APP) of the sum of haptoglobin, CRP and pig-MAP in healthy pigs ranged between 14 mg/kg

$BW^{0.75}$/day and 61 mg/kg $BW^{0.75}$/day (Table 6.2). In pigs with an activated immune system, the estimated APP synthesis of the sum of haptoglobin, CRP and pig-MAP ranged between 59 and 126 mg/kg $BW^{0.75}$/day (Table 6.2). Assuming that the APP synthesis of haptoglobin, CRP and pig-MAP is approximately half of the total APP synthesized, the estimated total APP synthesis of pigs with an activated immune system ranged between 117 and 251 mg/kg $BW^{0.75}$/day. The estimated difference in APP synthesis between healthy and challenged pigs was largest in PCV2 infected pigs, *i.e.* 112 mg/kg $BW^{0.75}$/day, compared to pigs in the studies described in Chapter 4 and 5.

Table 6.2 Serum acute phase protein (APP) concentration and estimated synthesis rate of three APP in healthy pigs and in pigs with an activated immune system[1].

		Healthy control pigs		**Immune system activated pigs**		**Difference in APP synthesis[2], mg/kg $BW^{0.75}$/d**
Challenge	**APP**	**Serum, mg/mL**	**APP synthesis, mg/kg $BW^{0.75}$/d**	**Serum, mg/mL**	**APP synthesis, mg/kg $BW^{0.75}$/d**	
Porcine circovirus type 2 field infection, n = 10 (Parra *et al.*, 2006)	haptoglobin	0.2	2.7	5.0	68.2	65.4
	CRP	0.005	0.4	0.14	12.3	11.9
	pig-MAP	0.8	10.7	3.3	45.2	34.5
	Sum		**14**		**126**	**112**
Contrast in health status, n = 25 (Chapter 4)	haptoglobin	1.3	15.9	2	24.4	8.5
	CRP	0.363	28.8	0.442	35.0	6.2
	pig-MAP	1.3	15.9	1.3	15.9	0.0
	Sum		**61**		**75**	**15**
CFA challenge, n = 16 (Chapter 5)	haptoglobin	0.7	8.9	1.9	24.7	15.8
	CRP	0.160	13.6	0.266	22.5	8.9
	pig-MAP	0.2	2.8	0.9	11.4	8.6
	Sum		**25**		**59**	**33**

[1]APP synthesis rate was estimated using equation 6.1 in the computer program SMART (Simulation and Modelling Assistant for Research and Training), to maintain serum plasma concentrations of haptoglobin, CRP and pig-MAP as observed in healthy pigs and pigs with an activated immune system. The pigs had an average bodyweight of 50 kg (Parra *et al.*, 2006), 25 kg (Chapter 4) and 40 kg (Chapter 5), respectively.
[2]Difference in APP synthesis rate between healthy pigs and pigs with an activated immune system.

Textbox II Calculations to determine the costs of APP production.

To quantify the AA use for APP synthesis during immune system activation we calculated the amount of AA that is required for a porcine APP response, and the amount of muscle protein that needs to be mobilized to fulfil this need. First the synthesis rate of haptoglobin, CRP and pigMAP were calculated for healthy pigs and for pigs with an activated immune system in three different scenarios (Table 6.2), *i.e.* induced by a porcine Circovirus infection in a commercial farm (Parra *et al.*, 2006), a contrast in health status (Chapter 4), or induced by a CFA challenge (Chapter 5). In the latter two scenarios only pigs fed a diet adequate in protein and AA were included. The synthesis rate of haptoglobin, CRP and pigMAP were calculated as the amount needed to sustain a particular pool size for APP (in mg), the latter calculated from measured serum APP concentrations and an assumed serum volume of 40 g/kg BW. In a steady state, APP synthesis equals APP decay, the latter calculated using the half-life of the APP. Therefore, APP synthesis (in mg/min) was calculated according to the equation below:

$$\text{APP synthesis} = APP_{t0} * (1\text{-}APP_{t0} * e^{-kt}),$$

Where APP_{t0} = the APP pool size (in mg) at time = 0, *i.e.* the time of interest at which a specific pool size should be maintained for each scenario, k=ln(2)/half-life (in min), and t = 1, *i.e.* the first minute, thus resulting in the amount of APP synthesized in the first minute needed to maintain initial pool size;

The half-life of APP was fixed at 19 h for CRP (Vigushin *et al.*, 1993), 132 h for haptoglobin (Dobryszycka *et al.*, 1979) and pigMAP, the latter value assumed to be similar as for haptoglobin, based on similarity in response pattern in time (Petersen, 2004).

To calculate the difference in the AA quantity required for an APP response between healthy pigs and pigs with an activated immune system using the three scenarios mentioned in Table 6.2, first the additional quantity of AA required for each APP, *i.e.* haptoglobin, CRP and pig-MAP was calculated. This was done by multiplying the estimated difference in APP synthesis of each of the three APP, by its AA composition (g/g) (Table IIa). Next, the sum of the difference in AA quantity required for the synthesis of these three APP was multiplied by 2, assuming that haptoglobin, CRP and pig-MAP include half of the amount of total APP synthesized. Finally, it was assumed that the AA required would have to be delivered by degrading muscle protein. Therefore, the quantity of muscle protein to be mobilized for the synthesis of APP was calculated for each AA as the total amount of that AA used for APP synthesis (in g/day), divided by the concentration of that AA in the *longissimus* muscle (LM)(g/g).

Table IIa Amino acid (AA) composition of acute phase proteins and pig muscle (g AA/kg protein).

AA	Hapto-globin[1]	CRP[1]	Pig-MAP[2]	SAA[1]	AGP[1]	Albu-min[3]	LM muscle protein[4]
Phe	30	105	46	103	64	50	40
Tyr	70	50	25	67	74	33	39
Trp	32	42	9	45	30	4	11
Leu	82	91	100	29	101	93	79
Ile	47	54	48	29	48	34	47
Val	84	77	81	18	46	59	51
Lys	92	71	49	33	75	100	85
His	38	16	26	35	17	20	30
Met	16	16	18	22	11	8	27
Cys	24	13	3	0	18	58	10
Thr	54	58	59	30	74	43	42
Arg	28	36	54	116	52	45	59
Pro	44	44	54	34	34	55	41
Gly	44	46	66	61	19	29	46
Ser	40	84	85	47	31	38	35
Ala	54	31	75	106	36	83	57

Abbreviations used: AGP, alpha- 1 acid glycoprotein; CRP, C-reactive protein; LM, *longissimus* muscle; pig-MAP, Pig major acute-phase protein; SAA, serum amyloid A.
[1]Reeds *et al.* (1994).
[2]The AA composition of Pig-MAP was calculated from the AA sequence (http://www.ncbi.nlm.nih.gov/protein/NP_001001537.1).
[3]Carlsson *et al.* (1977) except for Trp, *i.e.* Saifer and Palo (1969).
[4]Conde-Aguilera *et al.* (2010).

According to the calculations in pigs in Table 6.3, Trp will be the first limiting AA for APP synthesis when muscle protein is mobilized, followed by Cys and Val. The estimated increase in muscle protein breakdown rate, *i.e.* muscle protein that is mobilized to release AA for APP synthesis, in pigs with an activated immune system compared to healthy pigs was 96, 181 or 526 mg/kg $BW^{0.75}$/day for pigs in Chapter 4, 5 or for pigs in the study of Parra *et al.* (2006), respectively (Table 6.3). In LPS challenged chickens, the estimated amount of muscle protein mobilized to support APP assuming dietary AA are not available was approximately 1000 mg/kg$BW^{0.75}$/day (Barnes *et al.*, 2002). The reason why Trp may become first limiting instead of Phe as estimated in humans by Reeds *et al.* (1994), might be due to the slightly lower Phe concentration in haptoglobin and pig-MAP relative to the average concentration in APP used in the study of Reeds *et al.* (1994). Interestingly, APP seem to be also high in Ser, a non-essential AA, which is also the case for immunoglobulins and is suggested to be potentially important for the immune system (MacRae, 1993; Sandberg *et al.*, 2007).

Table 6.3 The calculated increase in the rate of AA utilization required for APP synthesis and increase in the muscle protein breakdown rate between healthy and challenged pigs.

AA_i	**Porcine circovirus type 2 in 50 kg pigs (Parra *et al.*, 2006)**		**Contrast in health status in 25 kg pigs (Chapter 4)**		**CFA challenge in 40 kg pigs (Chapter 5)**	
	Increase in use of AA_i for APP[1]	Increase in muscle protein breakdown for APP[2]	Increase in use of AA_i for APP[1]	Increase in muscle protein breakdown for APP[2]	Increase in use of AA_i for APP[1]	Increase in muscle protein breakdown for APP[2]
			mg/kg $BW^{0.75}$/day			
Phe	9.6	239	1.9	45	3.8	94
Tyr	12.1	310	1.9	47	3.8	94
Trp	5.9	526[3]	1.0	96[3]	1.9	181[3]
Leu	19.8	250	2.9	31	6.3	78
Ile	10.7	228	1.9	31	3.1	72
Val	18.4	362	1.9	47	5.7	111
Lys	17.0	201	2.9	29	5.0	61
His	7.2	238	1.0	28	1.9	67
Met	3.7	139	0.0	17	1.3	43
Cys	3.7	368	1.0	57	1.3	109
Thr	12.5	297	1.9	39	3.8	93
Arg	8.0	140	1.0	15	2.5	43
Pro	10.5	257	1.0	31	3.1	79
Gly	11.4	248	1.0	29	3.8	76
Ser	13.1	374	1.9	50	4.4	125
Ala	13.0	228	1.0	23	3.8	65

[1]The increase in the utilization rate of AA_i required for each APP was calculated by multiplying the estimated difference in APP synthesis (Table 6.2) by the AA composition of each APP. To estimate the total costs of AA for APP synthesis, it was assumed that haptoglobin, CRP, and pig-MAP represent half of the total amount of APP synthesized after immune system activation. The sum of the difference in AA_i quantity required for the synthesis of these three APP between healthy pigs and pigs with an activated immune system was therefore multiplied by 2.
[2]The rate of muscle protein breakdown (mg/kg $BW^{0.75}$d), needed to provide AA_i for APP synthesis was calculated by dividing the amount of AA_i required for APP (g/kg $BW^{0.75}$/day) by the LM muscle content of AA_i (g/g).
[3]The amount of muscle protein that is broken down for APP synthesis, with Trp being first limiting for APP synthesis is underlined.

In Chapter 5, a reduction in whole body protein deposition (6.25·N retention based on N retention measurements) was observed of 0.63 g/kg$BW^{0.75}$/day in pigs post compared to pre-challenge on a diet adequate in protein and AA. According to the calculations in the present Chapter, the increase in the rate of muscle protein breakdown was 0.181 g/kg$BW^{0.75}$/day to provide enough Trp for the increase in APP in CFA-challenged pigs. Thus, of the observed decrease in whole body protein deposition based on N retention measures, approximately 30% (0.181/0.63) could be attributed to the loss of muscle protein for incorporation of AA into APP (Figure 6.5). In Chapter 4, pigs characterized as having a low health status had a greater N retention when

expressed in g/(kg BW/day) than pigs characterized as having a high health status, thus the estimated quantity of muscle protein mobilized in order to supply AA for incorporation into APP was probably not high enough to affect protein deposition, or more likely, the observed compensatory growth counteracted the reduction in protein deposition related to APP synthesis.

In conclusion, these calculations indicate that of the reduction in protein deposition observed in pigs challenged with CFA (Chapter 5), approximately 30% (0.181/0.63) could be attributed to a reduction in protein deposition due to muscle mobilization for incorporation of AA into APP (Figure 6.5). Moreover, these calculations indicate that in pigs the utilisation of Trp, Ser, Cys and Val for APP synthesis may increase, whereas especially Lys and Leu may become excessive.

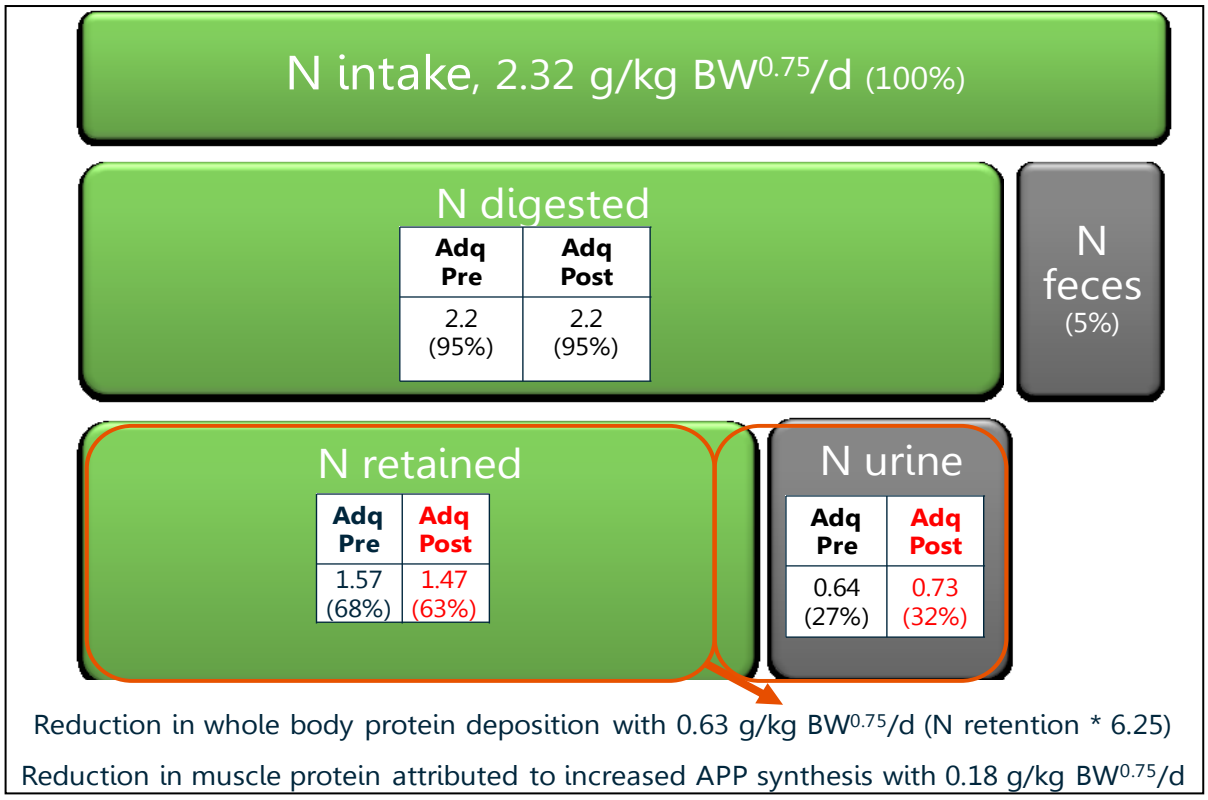


Figure 6.5 Reduction in protein deposition between unchallenged (pre) and CFA challenged (post) pigs fed a diet adequate in protein and AA (Adq) (Chapter 5) and estimated reduction in muscle protein deposition, attributable to an increased muscle protein breakdown for APP synthesis.

The difference in APP synthesis between high health and low health pigs in Chapter 4 may be an underestimate of what can be observed under commercial conditions, as the APP serum concentration and the estimated APP synthesis for healthy pigs was relatively high in the study described in Chapter 4, compared to unchallenged pigs in Chapter 5 and the unchallenged SPF pigs in commercial pig farms in the study of Parra *et al.* (2006). The CFA challenge in Chapter 5 and PCV2 infection in the study of Parra *et al.* (2006), are both examples of acute rather than chronic conditions of immune system activation. In PCV2 infected pigs haptoglobin concentrations increased by 2400% (Parra *et al.*, 2006), whereas in Chapter 5, haptoglobin concentrations increased by 170%. To put this in perspective, as depicted in Table 1.2, serum haptogobin

concentrations in pigs from commercial farms were increased compared to control to a similar extent as observed in Chapter 5, *e.g.* in case of a moderate inflammation induced by lower sanitary status and the absence of antibiotic-supplementation (150% greater compared to control, Le Floc'h *et al.* (2006)), in case of observed incidence of lung lesions at slaughter (380% greater compared to control, Pallarés *et al.*, 2008) or due to growth retardation (60% greater compared to control, Chen *et al.*, 2003). Moreover, commercial pigs can be continuously exposed to high levels of pathogen pressure suboptimal housing conditions, and other factors related to health status (Figure 1.3), which lead to chronic immune system activation. Compared to healthy commercially raised pigs, serum haptoglobin concentrations were greater in pigs with respiratory symptoms caused by *M. hyopneumoniae* infection (+1650%), or in pigs with signs of inflammation due to tail and ear bites, arthritis, rectal prolapse, or ulcerated umbilical hernia (+1900%; Parra *et al.*, 2006). In addition, Clapperton *et al.* (2005b) observed a negative correlation between serum APP concentrations and daily weight gain or feed intake in 18 and 24 weeks old pigs. In summary, the calculated increased utilisation of AA related to an APP response in CFA challenged pigs in Chapter 5 seems relevant for comparison with the chronic conditions of immune system activation observed in practice. The calculated reduction in protein deposition related to APP synthesis was 181 mg/kg $BW^{0.75}$/day in CFA challenged pigs compared to unchallenged pigs (Table 6.3). This is equivalent to a difference in ADG of 20 g/day in pigs with a BW of 50 kg, assuming that net protein deposition rate can be calculated as 0.17 · ADG. Considering the variation in APP in pigs between commercial farms, this may even be an underestimate. In the studies described in Chapter 4 and 5, the feed intake was kept similar between healthy and immune challenged pigs. Therefore no estimate on the costs in relation to a reduction in feed intake could be made. In a situation of poor housing conditions, *i.e.* poor hygiene conditions, exposure to extreme temperatures and limited floor space allowance as described in the study of Pastorelli *et al.* (2012), the ADG was reduced with 16%, of which ¼ was related to an increased maintenance requirement and ¾ related to a reduction in feed intake. In contrast, in case of respiratory infections a reduction in ADG of 25% was observed, of which almost ¾ was related to an increased maintenance requirement. The observed variation in ADG between commercial pig farms ranged between 570 and 930 g/day based on data from 887 Dutch pig farms (Figure 1.1) (AgroVision, 2012), *i.e.* a difference in ADG of almost 360 g/day. In this way, the reduction in ADG attributed to the APP response as observed in the study in Chapter 5, *i.e.* related to increased maintenance requirements for AA, would explain 6% (20/360) of the observed difference in mean ADG between Dutch farms. Considering these findings, the reduction in feed intake is likely a major contributor to the reduction in ADG during chronic immune system activation as a result of poor housing conditions, whereas an increase in maintenance requirement for AA associated with an APP response is expected to be a major contributor to the reduction in ADG in case of systemic inflammations as observed in case of respiratory diseases.

Associated metabolic changes related to immune system activation

The increase in breakdown of muscle protein is part of the associated metabolic costs from catabolic processes, including a reduction in feed intake (Williams *et al.*, 1997a; Johnson, 1998; Sandberg *et al.*, 2006; Daiwen *et al.*, 2008; Pastorelli *et al.*, 2012), a decrease in protein synthesis (Zamir *et al.*, 1992; Breuille, 1999), and an increased deamination of glucogenic AA (Klasing and Johnstone, 1991; Lochmiller and Deerenberg, 2000; Le Floc'h *et al.*, 2004). These associated metabolic processes together can contribute to the reduction in protein deposition under conditions in which the immune system is activated. Moreover, it is suggested that besides cytokines, an increased glucocorticoid production by the adrenal gland released to the blood seems to play a major role in regulating muscle protein breakdown during inflammation (Hasselgren, 2000; Schakman *et al.*, 2012). Glucocorticoids induce muscle protein breakdown during fasting, whereas insulin blocks this response in the fed state (Lecker *et al.*, 1999). In line with our findings, Klasing (2013) suggested that a decreased appetite, an increased metabolic rate and metabolic inefficiencies dominate the nutritional costs of responses of the immune system.

Optimal AA profile for growth *vs.* immunity

In order to obtain insight in the optimal dietary AA profile for body protein deposition for growth and for immune system functioning, the estimated difference in AA utilization for APP production (Table 6.3) was combined with the observed changes in protein deposition and ILR of AA in pigs challenged with CFA (Chapter 5).

The calculated increase in the rate of AA utilization required for APP synthesis (Table 6.3) was 5.0 mg /kg$^{0.75}$/day for Lys in CFA challenged pigs (Chapter 5; Table 6.3), whereas an additional 15.3 mg Lys /kg$^{0.75}$/day is released due to the increased mobilization of body protein of 181 mg/kg BW$^{0.75}$/day (Table 6.3). These estimated changes in AA utilization due to their increased incorporation into APP and increased oxidation of excessive AA are displayed for each AA in pigs fed an adequate protein and AA diet (Figure 6.5a) and a diet restricted in protein and AA (Figure 6.5b). The change in AA utilization related to protein deposition based on the observed N retention measurements in Chapter 5, were calculated by multiplying the observed reduction in protein deposition (0.63 g/kg BW$^{0.75}$/day), post-challenge compared to pre-challenge, by the AA composition of whole body protein (Kyriazakis *et al.*, 1993). The changes in ILR between pre- and post-challenge day 3 and 8 are expressed in mg/kg BW$^{0.75}$/day (Figure 6.5c and d).

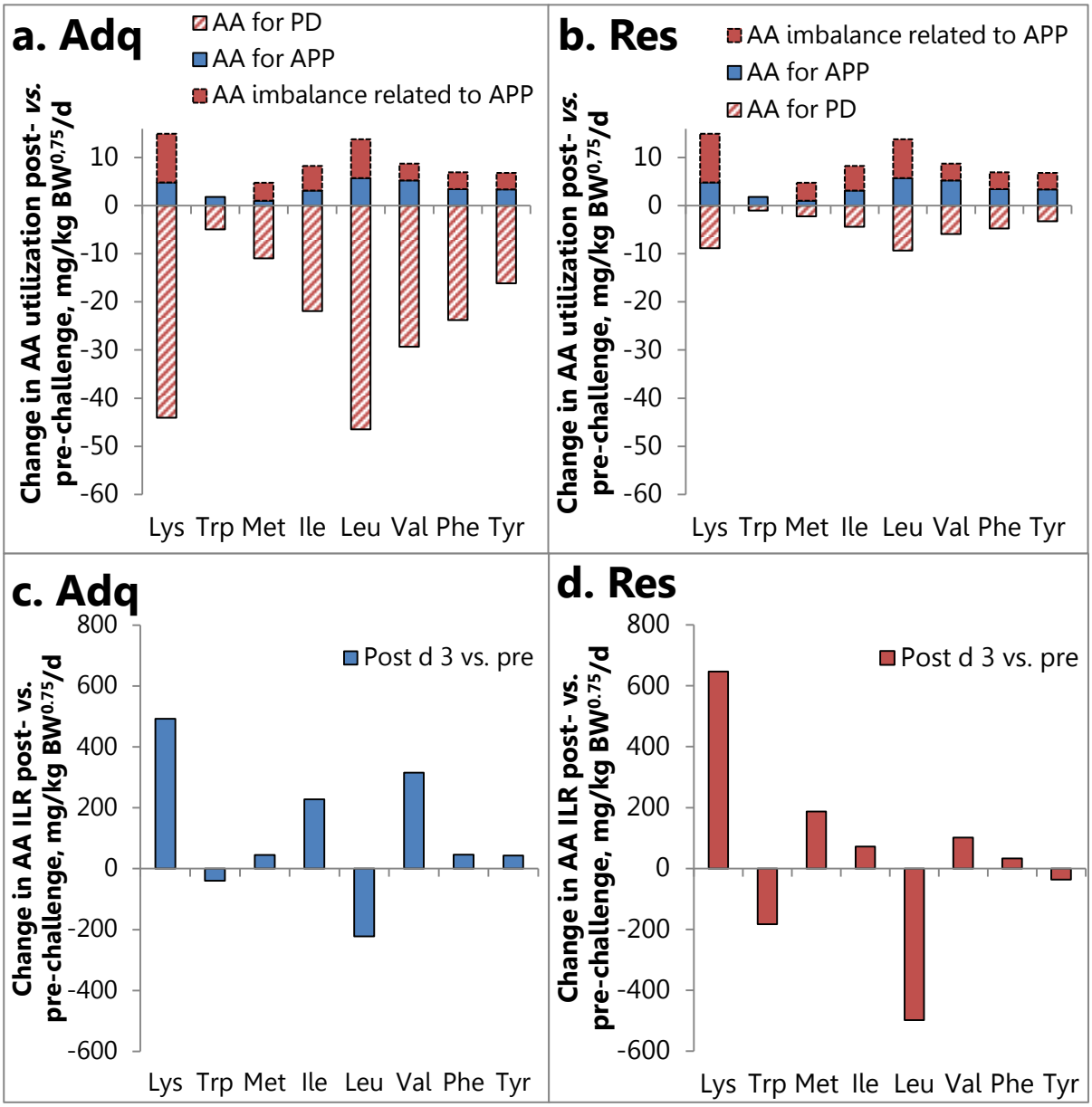


Figure 6.5 The estimated changes in utilization of AA for protein deposition, based on N retention measurements, and for synthesis of APP, based on the difference in APP concentrations in blood on day 3 post-challenge (panel a and b) and in ILR of AA in pigs fed an adequate (Adq) and Restricted (Res) protein and AA diet (panel c and d) as affected by immune challenge (post- *vs.* pre-challenge) (Chapter 5)[1,2].

[1]The calculated increase in the rate of AA utilization for APP synthesis from Table 6.3, are displayed, and the release of excessive AA, *i.e.* the release of AA due to the increased mobilization of 181 mg body protein/kg $BW^{0.75}$/day minus the use of AA for incorporation into APP (Table 6.3).

[2]The changes in AA utilization related to protein deposition as based on the observed change in N retention, were calculated by multiplying the observed reduction in protein deposition post-challenge compared to pre-challenge by the AA composition of whole body protein (Kyriazakis *et al.*, 1993).

The ideal protein concept is underlined by the idea that there is an optimal pattern of dietary AA that correspond to the AA requirements of the animal, and the requirement

for all individual essential AA are expressed relative to the requirement for Lys as it is often the first limiting AA for body protein deposition for growth (Ball *et al.*, 2007). In order to provide insight in the optimal dietary AA profile for body protein deposition and for immune system functioning of growing pigs, first the effect of immune system activation on Lys utilisation and requirement are discussed. The ILR for Lys was greater post-challenge compared to pre-challenge, which is likely related to a greater oxidation of Lys, considering the numerically lower pool size post-challenge, the lower body protein deposition, and the suggested excessive release of Lys from muscle protein mobilization for APP synthesis and resulting AA imbalance, especially in pigs fed a diet with restricted protein and AA levels (Fig 6.5b and d). Lys transport from muscle to the blood is facilitated in severe burn patients, comparable with the changes in whole body protein breakdown and synthesis observed in sepsis or trauma (Biolo *et al.*, 2002). Based on literature it is likely that the absolute AA requirement for Lys decreases during immune system activation, as a consequence of a reduction in maximum protein deposition (Klasing and Barnes, 1988; Williams *et al.*, 1997a,b,c; Webel *et al.*, 1998).

The ILR of Ile and Val are suggested to behave similar to that of Lys, their increase is suggested to be related to an increased oxidation as a result of AA imbalance. For Val, however, a greater utilisation for APP may also be likely, considering the greater numeric reduction in Val pool size compared to the change in pool size for Lys. In Chapter 4, the ILR for Ile tended to be higher in pigs with a low health status compared to pigs with a high health status, yet the occurrence of compensatory growth in these pigs fed an adequate AA supply complicated the interpretation of results (Chapter 4).

The observed changes in ILR for Met, Phe and Tyr after immune system stimulation may be related to greater oxidation of these AA. The plasma pool size of Met was significantly greater at day 8 post-challenge compared to day 3 in pigs fed the adequate diet, which suggests a greater oxidation of Met, likely related to an increased conversion to Cys (Chapter 5). The requirement for sulphur AA expressed as SID sulphur AA: Lys is suggested to increase from 0.55 to 0.75 in pigs following an *i.m.* LPS challenge or by continuous pathogen exposure under commercial conditions (Kim *et al.*, 2012). Other studies in pigs revealed that immune system activation by *i.m.* LPS administration increases the optimal dietary Met to Met + Cys ratio (Litvak *et al.*, 2013). Rakhshandeh *et al.* (2014), however, estimated that the SID sulphur AA requirement in g/day to maximize protein deposition in LPS challenged pigs decreased with 8%, without observing an effect on efficiency of utilisation of Cys for body protein deposition. The authors suggested that immune system activation increased the maintenance requirements for sulphur AA as indicated by the change in intercept at zero protein deposition in a dose-response study.

The ILR for Tyr and release of Tyr from breakdown tended to be lower at day 8 compared to day 3 post-challenge, which may be related to decreased conversion of Phe to Tyr during inflammation (Capuron *et al.*, 2011; Chapter 5).

The ILR for Trp was numerically lower at day 3 post-challenge compared to pre-challenge, and the pool size of Trp was similar at day 3 compared to pre-challenge in

pigs fed the adequate protein and AA diet and lower in pigs fed the restricted diet (Figure 6.5c and d), most likely indicating that Trp is the limiting AA post-challenge. The former is in line with the calculations in this thesis that suggest that Trp becomes limiting for APP synthesis. In a study of Le Floc'h (2010), the dietary Trp requirement expressed in g/kg to maximize ADG was not affected by moderate inflammation due to modification in housing conditions, and was suggested to be related to a decreased feed intake. In the latter study no difference in slope of the response in ADG to an increase in dietary Trp intake was observed between piglets suffering from moderate inflammation compared to unchallenged control, indicating that the efficiency in Trp utilization for body protein retention (growth) was not affected. In contrast, a reduced efficiency of Trp utilization for body protein deposition was observed in pigs challenged with LPS, which is possibly related to the increased oxidation of Trp through the IDO pathway to kynurenine (de Ridder *et al.*, 2012).

The numeric lower ILR for Leu post-challenge compared to pre-challenge is in line with the lower body protein deposition and associated change in AA utilisation (Figure 6.5 a and b), also considering the high proportion of Leu in whole body protein. Although the numeric lower pool size of Leu post-challenge compared to pre-challenge, may suggests that Leu becomes limiting for protein deposition, this seems unlikely. Leu is expected to become excessive rather than limiting for protein deposition, considering the reduction in body protein retention and the expected increase in Leu oxidation due to mobilization of muscle protein for APP synthesis, as estimated in Table 6.3. As the ILR of AA reflects the sum of AA needed for protein synthesis and AA oxidation, possibly the decrease in protein synthesis is outweighed by the increase in oxidation in case of Leu. Vaccination with diphtheria, pertussis and tetanus as an immune challenge in human raised the Leu oxidation and whole-body protein breakdown with 25 and 20%, respectively, as measured in the isotopic enrichment in breath and blood following ^{13}C labelled Leu and $NaH^{13}CO_3$ administration. No measurements on fluxes of other AA were made in their study (Kurpad *et al.*, 1999).

Unfortunately the ILR for Cys and Thr could not be determined in the studies described in the present thesis, although these AA are likely involved in immune system functioning.

In conclusion, based on the calculations in the present thesis on muscle mobilization of AA for APP synthesis and their metabolic consequences, and the observed changes in ILR of a number of essential AA, it seems that in particular Trp may become limiting for immune system functioning, whereas Lys becomes excessive. Furthermore, indications were found that the utilization of Met, Tyr and Val for immune system functioning increases in pigs with an activated immune system.

General conclusions

- The health status web that incorporates farm data on average daily gain, energy conversion ratio, mortality, the incidence of pleuritis, and the incidence of lung and liver abnormalities at slaughter is a useful tool to categorize growing-finishing pig farms on the basis of their health status (Chapter 2).
- The dose-response technique to determine amino acid requirements using a decreasing dietary Lys supply strategy in time and a step length of 3 day with urinary N excretion as response criterion provides a simple, accurate technique to quantitatively estimate changes in amino acid requirements of individual meal-fed pigs (Chapter 3).
- Pigs respond metabolically faster to an increase than to a decrease in a dietary amino acid imbalance. This needs to be taken into account when determining amino acid requirements of individual animals using a dose-response approach (Chapter 3).
- The isotope dilution technique is appropriate for measuring changes in the metabolic utilization of various essential amino acids simultaneously and only requires short term *in vivo* measurements. This makes the technique appropriate for situations in which it is difficult to maintain a steady state *e.g.* in case of studying the effects of immune system activation (Chapter 4 and 5).
- The occurrence of compensatory gain in pigs from a farm characterized as having a low status, proves that it is difficult to maintain a contrast in health status, and that pigs can adapt quickly to a change in housing conditions (Chapter 4).
- The magnitude of the reduction in growth performance by immune system activation depends on the nature of the challenge. In the absence of effects on feed intake, health challenging conditions may affect performance due to alterations in post-absorptive amino acid metabolism, as also indicated by increased urinary N losses, and a tendency for a reduced N retention and a lower utilization of digestible N for N retention in pigs with a systemic inflammation (Chapter 5), or by a reduction in faecal nutrient digestibility as indicated for dry matter and N in pigs from a farm with a low health status (Chapter 4).
- The observed changes in protein and amino acid metabolism after immune stimulation imply that especially tryptophan may become limiting during immune system activation, whereas lysine becomes excessive (General Discussion). Furthermore, the utilization of methionine, tyrosine, and valine for immune system functioning increases in pigs with an activated immune system, associated with an elevation in serum concentrations of acute phase proteins (Chapter 5).
- A dietary amino acid or protein supply can modulate the acute phase response pre- and post-challenge, stressing the importance of an adequate dietary amino acid supply for appropriate functioning of the immune system of growing-finishing pigs (Chapter 4 and 5).

Directions for farm specific feeding and recommendations

- Directions for farm specific feeding:
    - The health status web (Chapter 2) can be of use for feed manufacturers to develop targeted strategies to accommodate the nutritional requirements of pigs belonging to groups of farms sharing a particular health status, in turn improving efficiency of pig production.
    - Current requirement estimates for growing-finishing pigs are formulated to maximize protein deposition for growth and do not take into account the increased utilization of amino acids for immune functioning as induced by health challenging conditions. Reductions in performance in commercial farms likely arise both from acute inflammation triggered by infections and due to low grade sustained inflammation caused by poor sanitary conditions. In both cases, an adequate supply of dietary amino acids is required for immune system functioning and for body protein deposition for growth. Before implementing targeted feeding strategies for farms sharing a common health status, future research should be conducted to study the possible beneficial effects of increasing the dietary supply of particularly tryptophan, methionine, tyrosine, and valine relative to lysine for immune system function and for body protein deposition in pigs from farms with a different health status. In contrast, in pigs from a farm with a high health status, a deficient level of dietary methionine + cysteine, threonine and tryptophan, did not impair body weight gain, and N retention (Chapter 4). Thus, further optimization of diets for pigs from farms with a high health status may also be required.
    - Feeding strategies should not be aimed at preventing protein breakdown during disease, but at providing sufficient amino acids for body protein synthesis for growth and immune functioning.
- Non-invasive techniques to determine the amino acid requirement of pigs from farms with a different health status may include a simplified dose-response technique (Chapter 3) using an oral dose of ^{15}N urea and determination of ^{15}N enrichment in saliva as a response parameter, and the detection of APP concentrations in saliva instead of blood.

References

Aarestrup FM, Duran CO, Burch DGS (2008) Antimicrobial resistance in swine production. *Anim Health Res Rev* **9**, 135-148.

Adams CA (2006) Nutrition-based health in animal production. *Nutr Res Rev* **19**, 79-89.

AgroVision (2011) Bedrijfsvergelijking AgroVision B.V. *Kengetallenspiegel Jan-Dec 2011*. The Netherlands (in Dutch).

AgroVision (2012) Bedrijfsvergelijking AgroVision B.V. *Kengetallenspiegel Jan-Dec 2012*. The Netherlands (in Dutch).

Amory JR, Mackenzie AM, Eckersall PD, Stear MJ, Pearce GP (2007) Influence of rearing conditions and respiratory disease on haptoglobin levels in the pig at slaughter. *Res Vet Sci* **83**, 428-435.

Andreasen M, Mousing J, Thomsen LK (2001) No simple association between time elapsed from seroconversion until slaughter and the extent of lung lesions in Danish swine. *Prev Vet Med* **52**, 147-161.

ARC (1981) *The nutrient requirements of pigs*. Slough, UK: Commonwealth Agricultural Bureaux.

Asai T, Mori M, Okada M, Uruno K, Yazawa S, Shibata I (1999) Elevated serum haptoglobin in pigs infected with porcine reproductive and respiratory syndrome virus. *Vet Immunol Immunop* **70**, 143-148.

Ball RO, Urschel KL, Pencharz PB (2007) Nutritional consequences of interspecies differences in arginine and lysine metabolism. *J Nutr* **137**, 1626S-1641S.

Barnes DM, Song Z, Klasing KC, Bottje W (2002) Protein metabolism during an acute phase response in chickens. *Amino Acids* **22**, 15-26.

Batterham ES, Andersen LM, Baigent DR, White E (1990) Utilization of ileal digestible amino acids by growing pigs: effect of dietary lysine concentration on efficiency of lysine retention. *Br J Nutr* **64**, 81-94.

Baumann H, Gauldie J (1994) The acute phase response. *Immunol Today* **15**, 74-80.

Beisel WR (1996) Nutrition and immune function: overview. *J Nutr* **126**, 2611S-2615S.

Bertolo RF, Moehn S, Pencharz PB, Ball RO (2005) Estimate of the variability of the lysine requirement of growing pigs using the indicator amino acid oxidation technique. *J Anim Sci* **83**, 2535-2542.

Beutler B (2004) Innate immunity: an overview. *Mol Immunol* **40**, 845-859.

Bibby J, Toutenburg H (editors)(1977) *Prediction and improved estimation in linear models.* Chichester, UK: John Wiley and Sons.

Bikker P, Verstegen MWA, Campbell RG, Kemp B (1994) Digestible lysine requirement of gilts with high genetic potential for lean gain, in relation to the level of energy intake. *J Anim Sci* **72**, 1744-1753.

Bikker P, Verstegen MWA, Campbell RG (1996a) Performance and body composition of finishing gilts (45 to 85 kilograms) as affected by energy intake and nutrition in earlier life: II. Protein and lipid accretion in body components. *J Anim Sci* **74**, 817-826.

Bikker P, Verstegen MWA, Kemp B, Bosch MW (1996b) Performance and body composition of finishing gilts (45 to 85 kilograms) as affected by energy intake and nutrition in earlier life: I. Growth of the body and body components. *J Anim Sci* **74**, 806-816.

Biolo G, Fleming RYD, Maggi SP, Nguyen TT, Herndon DN, Wolfe RR (2002) Inverse regulation of protein turnover and amino acid transport in skeletal muscle of hypercatabolic patients. *J Clin Endocrinol Metabol* **87**, 3378-3384.

Biolo G, Toigo G, Ciocchi B, Situlin R, Iscra F, Gullo A, Guarnieri G (1997) Metabolic response to injury and sepsis: changes in protein metabolism. *Nutr* **13**, 52-57.

Bishop SC, Axford RFE, Nicholas FW, Owen JB (editors)(2010) *Breeding for disease resistance in farm animals*. 3rd ed., pp.156-157. Oxfordshire, UK: CABI.

Boersma WJA, van der Meulen J, Niewold TA (2009) Balance between porcine disease and welfare. In *The welfare of pigs,* Vol. 7, pp. 239-240 [JN Marchant-Forde, editor]. Dordrecht, The Netherlands: Springer Science.
Bonde M, Toft N, Thomsen PT, Sørensen JT (2010) Evaluation of sensitivity and specificity of routine meat inspection of Danish slaughter pigs using Latent Class Analysis. *Prev Vet Med* **94**, 165-169.
Bondt N, Puister L, Ge L, van der Veen H, Bergevoet R, Douma B, van Vliet A, Wehling K (2012) *Trends in veterinary antibiotic use in the Netherlands 2004-2012*. Wageningen, The Netherlands: LEI Wageningen UR.
Bredt DS, Snyder SH (1994) Nitric oxide: a physiologic messenger molecule. *Ann Rev Biochem* **63**, 175-195.
Breuillé D, Rose F, Arnal M, Melin C, Obled C (1994) Sepsis modifies the contribution of different organs to whole-body protein synthesis in rats. *Clin Sci* **86**, 663-669.
Breuillé D, Voisin L, Contrepois M, Arnal M, Rose F, Obled C (1999) A sustained rat model for studying the long-lasting catabolic state of sepsis. *Infect Immunol* **67**, 1079-1085.
Breuillé D, Béchereau F, Buffière C, Denis P, Pouyet C, Obled C (2006) Beneficial effect of amino acid supplementation, especially cysteine, on body nitrogen economy in septic rats. *Clin Nutr* **25**, 634-642.
Bronte V, Zanovello P (2005) Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* **5**, 641-654.
Buchanan JB, Johnson RW (2007) Regulation of food intake by inflammatory cytokines in the brain. *Neuroendocrin* **86**, 183-190.
Burger D, Dayer J-M (2002) High-density lipoprotein-associated apolipoprotein A-I: the missing link between infection and chronic inflammation? *Autoimmun Rev* **1**, 111-117.
Calder PC (2006) Branched-chain amino acids and immunity. *J Nutr* **136**, 288S-293S.
Calder PC (2007a) Immunological parameters: What do they mean? *J Nutr* **137**, 773S-780S.
Calder PC (2007b) Immunonutrition in surgical and critically ill patients. *Br J Nutr* **98**, 133S-139S.
Calder PC, Yaqoob P (2012) Nutrient regulation of the immune response. In *Present knowledge in nutrition*, 10th ed., pp. 688-708 [JW Erdman, IA Macdonald and SH Zeisel, editors]. Chichester, UK: Wiley-Blackwell.
Campbell RG, Taverner MR, Curic DM (1984) Effect of feeding level and dietary protein content on the growth, body composition and rate of protein deposition in pigs growing from 45 kg to 90 kg. *Anim Prod* **38**, 233-240.
Capuron L, Schroecksnadel S, Féart C, Aubert A, Higueret D, Barberger-Gateau P, Layé S, Fuchs D (2011) Chronic low-grade inflammation in elderly persons is associated with altered tryptophan and tyrosine metabolism: role in neuropsychiatric symptoms. *Biol Psychiatry* **70**, 175-182.
Carlsson RNK, Ingvarsson BI, Börje W (1977) Isolation and characterization of albumin from porcine serum, colostrum and urine. *Int J Biochem* **8**, 285-294.
Carpintero R, Piñeiro M, Andrés M, Iturralde M, Alava MA, Heegaard PMH, Jobert JL, Madec F, Lampreave F (2005) The concentration of apolipoprotein A-I decreases during experimentally induced acute-phase processes in pigs. *Infect Immunol* **73**, 3184-3187.
Carroll JA, Fangman TJ, Hambach AK, Wiedmeyer CE (2004) The acute phase response in pigs experimentally infected with *Escherichia coli* and treated with systemic bactericidal antibiotics. *Livest Prod Sci* **85**, 35-44.
Centraal Veevoederbureau (1996) *Requirements for amino acids in piglets and pigs*. CVB Report 14. Lelystad, The Netherlands: CVB.
Centraal Veevoederbureau (2004) *Table on feed ingredients. Data on the chemical composition, digestibility and nutritional value of feedstuffs*. Lelystad, The Netherlands: CVB.
Centraal Veevoederbureau (2008) *Tabellenboek Veevoeding*. CVB-reeks nr 46. Lelystad, The Netherlands: CVB.
Chen H, Lin J, Fung H, Chu R (2003) Serum acute phase proteins and swine health status. *Can J Vet Res* **67**, 283-290.

Chen DW, Zhang KY, Wu CY (2008) Influences of lipopolysaccharide-induced immune challenge on performance and whole-body protein turnover in weanling pigs. *Livest Sci* **113**, 291-295.
Chen Y, Guillemin GJ (2009) Kynurenine pathway metabolites in humans: disease and healthy states. *Int J Tryptophan Res* **2**, 1-19.
Chen Y, Chen D, Tian G, He J, Mao X, Mao Q, Yu B (2012) Dietary arginine supplementation alleviates immune challenge induced by Salmonella enterica serovar Choleraesuis bacterin potentially through the Toll-like receptor 4-myeloid differentiation factor 88 signalling pathway in weaned piglets. *Br J Nutr* **108**, 1069-1076.
Chipponi JX, Bleier JC, Santi MT, Rudman D (1982) Deficiencies of essential and conditionally essential nutrients. *Am J Clin Nutr* **35**, 1112-1116.
Choi YK, Goyal SM, Joo HS (2003) Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. *Can Vet J* **44**, 735-737.
Cid MC, Grant DS, Hoffman GS, Auerbach R, Fauci AS, Kleinman HK (1993) Identification of haptoglobin as an angiogenic factor in sera from patients with systemic vasculitis. *J Clin Invest* **91**, 977-985.
Clapperton M, Bishop SC, Cameron ND, Glass EJ (2005a) Associations of weight gain and food intake with leukocyte sub-sets in Large White pigs. *Livest Prod Sci* **96**, 249-260.
Clapperton M, Bishop SC, Cameron ND, Glass EJ (2005b) Associations of acute phase protein levels with growth performance and with selection for growth performance in Large White pigs. *Anim Sci* **81**, 213-220.
Clapperton M, Glass EJ, Bishop SC (2008) Pig peripheral blood mononuclear leucocyte subsets are heritable and genetically correlated with performance. *Animal* **2**, 1575-1584.
Colditz IG (2002) Effects of the immune system on metabolism: implications for production and disease resistance in livestock. *Livest Prod Sci* **75**, 257-268.
Coma J, Carrion D, Zimmerman DR (1995) Use of plasma urea nitrogen as a rapid response criterion to determine the lysine requirement of pigs. *J Anim Sci* **73**, 472-481.
Conde-Aguilera JA, Barea R, Le Floc'h N, Lefaucheur L, van Milgen J (2010) A sulfur amino acid deficiency changes the amino acid composition of body protein in piglets. *Animal* **4**, 1349-1358.
Conway EJ (editor) (1962) *Microdiffusion analysis and volumetric error*. 3rd ed., pp. 430-434. London: Crosby Lockwood and Son Ltd.
Cray C (2012) Acute phase proteins in animals. In *Progress in molecular biology and translational science animal models of molecular pathology,* Vol. 105, pp. 114-138 [PM Conn, editor] San Diego, USA: Academic Press.
Cray C (2012) Acute phase proteins in animals. *Prog Mol Biol Transl Sci* **105**, 114-138.
Cray C, Zaias J, Altman NH (2009) Acute phase response in animals: a review. *Comp Med* **59**, 517-526.
Critser DJ, Miller PS, Lewis AJ (1995) The effects of dietary protein concentration on compensatory growth in barrows and gilts. *J Anim Sci* **73**, 3376-3383.
Cromwell GL (2002) Why and how antibiotics are used in swine production. *Anim Biotechnol* **13**, 7-27.
Daiwen C, Keying Z, Chunyan W (2008) Influences of lipopolysaccharide-induced immune challenge on performance and whole-body protein turnover in weanling pigs. *Livest Sci* **113**, 291-295.
Dalmau A, Temple D, Rodriguez P, Llonch P, Velarde A, (2009) Application of the Welfare Quality protocol at pig slaughter houses. *Anim Welf* **18**, 497-505.
Das TK, Waterlow JC (1974) The rate of adaptation of urea cycle enzymes, aminotransferases and glutamic dehydrogenase to changes in dietary protein intake. *Br J Nutr* **32**, 353-373.
Davis BL, Fraser JN, Burkey TE, Skjolaas KA, Dritz SS, Johnson BJ, Minton JE (2010) Oral inoculation with Salmonella enterica serovar Typhimurium or Choleraesuis promotes divergent responses in the somatotropic growth axis of swine. *J Anim Sci* **88**, 1642-1648.
de la Fuente H, Cibrián D, Sánchez-Madrid F (2012) Immunoregulatory molecules are master regulators of inflammation during the immune response. *FEBS Lett* **586**, 2897-2905.

de Lange CFM, Sauer WC, Souffrant W (1989) The effect of protein status of the pig on the recovery and amino acid composition of endogenous protein in digesta collected from the distal ileum. *J Anim Sci* **67**, 755-762.
de Ridder K, Levesque CL, Htoo JK, de Lange CFM (2012) Immune system stimulation reduces the efficiency of tryptophan utilization for body protein deposition in growing pigs. *J Anim Sci* **90**, 3485-3491.
Defa L, Changting X, Shiyan Q, Jinhui Z, Johnson EW, Thacker PA (1999) Effects of dietary threonine on performance, plasma parameters and immune function of growing pigs. *Anim Feed Sci Tech* **78**, 179-188.
DeLeve LD, Kaplowitz N (1991) Glutathione metabolism and its role in hepatotoxicity. *Pharmacol Ther* **52**, 287-305.
Dewey CE, Straw BE (2006) Herd examination. In *Diseases of swine*, 9th ed., pp. 3-14 [BE Straw, JJ Zimmerman, S D'Allaire and DJ Taylor, editors]. Ames, USA: Blackwell Publishing.
Di Lorenzo M, Bass J, Krantis A (1995) Use of L-arginine in the treatment of experimental necrotizing enterocolitis. *J Pediatr Surg* **30**, 235-241.
Diack AB, Gladney CD, Mellencamp MA, Stear MJ, Eckersall PD (2011) Characterisation of plasma acute phase protein concentrations in a high health boar herd. *Vet Immunol Immunop* **139**, 107-112.
Dobryszycka W, Osada J, Woźniak M (1979) Metabolic studies on hybrid haptoglobins. *Int J Biochem* **10**, 75-79.
Dolman MA, Vrolijk HCJ, de Boer IJM (2012) Exploring variation in economic, environmental and societal performance among Dutch fattening pig farms. *Livest Sci* **149**, 143-154.
Doumas BT, Bayse DD, Carter RJ, Peters T, Schaffer, R (1981) A candidate reference method for determination of total protein in serum I. Development and validation. *Clin Chem* **27**, 1642-1650.
Eckersall P (2000) Recent advances and future prospects for the use of acute phase proteins as markers of disease in animals. *Rev Med Vet* **151**, 577-584.
Edwards JF, Slauson DO (1983) Complete Freund's adjuvant-induced pneumonia in swine: A model of interstitial lung disease. *J Comp Pathol* **93**, 353-361.
Elbers ARW, Tielen MJM, Snijders JMA, Cromwijk WAJ, Hunneman WA (1992) Epidemiological studies on lesions in finishing pigs in the Netherlands. I. Prevelance, seasonality and interrelationship. *Prev Vet Med* **14**, 217-231.
Enøe C, Christensen G, Andersen S, Willeberg P (2003) The need for built-in validation of surveillance data so that changes in diagnostic performance of *post-mortem* meat inspection can be detected. *Prev Vet Med* **57**, 117-125.
Escobar J, van Alstine WG, Baker DH, Johnson RW (2004) Decreased protein accretion in pigs with viral and bacterial pneumonia is associated with increased myostatin expression in muscle. *J Nutr* **134**, 3047-3053.
European Parliament and the Council of the European Union: Regulation(EC) No. 854/2004 of the European Parliament and of the Council of 29, April 2004. Official Journal of the European Union, Luxembourg 2004, Annex I.
Fabian J, Chiba LI, Frobish LT, McElhenney WH, Kuhlers DL, Nadarajah K (2004) Compensatory growth and nitrogen balance in grower-finisher pigs. *J Anim Sci* **82**, 2579-2587.
Fablet C, Marois-Crehan C, Simon G, Grasland B, Jestin A, Kobisch M, Madec F, Rose N (2012) Infectious agents associated with respiratory diseases in 125 farrow-to-finish pig herds: A cross-sectional study. *Vet Microbiol* **157**, 152-163.
Fagbemi BO, Otesile EB, Makinde MO, Akinboade OA (1990) The relationship between dietary energy levels and the severity of *Trypanosoma brucei* infection in growing pigs. *Vet Parasitol* **35**, 29-42.
Fallarino F, Grohmann U, You S, McGrath BC, Cavener DR, Vacca C, Orabona C, Bianchi R, Belladonna ML, Volpi C, Santamaria P, Fioretti MC, Puccetti P (2006) The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. *J Immunol Meth* **176**, 6752-6761.

Fan MZ, Sauer WC, Hardin RT, Lien KA (1994) Determination of apparent ileal amino acid digestibility in pigs: effect of dietary amino acid level. *J Anim Sci* **72**, 2851-2859.
Fanali G, di Masi A, Trezza V, Marino M, Fasano M, Ascenzi P (2012) Human serum albumin: From bench to bedside. *Mol Aspects Med* **33**, 209-290.
Faure M, Mettraux C, Moennoz D, Godin JP, Vuichoud J, Rochat F, Breuille D, Obled C, Corthesy-Theulaz I (2006) Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. *J Nutr* **136**, 1558-1564.
Fern EB, Garlick PJ, Waterlow JC (1985) The concept of the single body pool of metabolic nitrogen in determining the rate of whole-body nitrogen turnover. *Hum Nutr Clin Nutr* **39**, 85-99.
Fournier T, Medjoubi-N N, Porquet D (2000) Alpha-1-acid glycoprotein. *Biochim Biophys Acta* **1482**, 157-171.
Fraile L, Alegre A, Lopez-Jimenez R, Nofrarias M, Segales J (2010) Risk factors associated with pleuritis and cranio-ventral pulmonary consolidation in slaughter-aged pigs. *Vet J* **184**, 326-333.
Frank JW, Mellencamp MA, Carroll JA, Boyd RD, Allee GL (2005) Acute feed intake and acute-phase protein responses following a lipopolysaccharide challenge in pigs from two dam lines. *Vet Immunol Immunopathol* **107**, 179-187.
Fuller MF, Cadenhead A, Mollison G, Sève B (1987) Effects of the amount and quality of dietary protein on nitrogen metabolism and heat production in growing pigs. *Br J Nutr* **58**, 277-285.
Gabay C, Kushner I, (1999) Acute-phase proteins and other systemic responses to inflammation. *New Engl J Med* **340**, 448-454.
Gabler NK, Spurlock ME (2008) Integrating the immune system with the regulation of growth and efficiency. *J Anim Sci* **86**, 64E-74E.
Gatnau R, Zimmerman DR, Nissen SL, Wannemuehler M, Ewan RC (1995) Effects of excess dietary leucine and leucine catabolites on growth and immune responses in weanling pigs. *J Anim Sci* **73**, 159-165.
Gershoff SN, Gill TJ, Simonian SJ, Steinberg AI (1968) Some effects of amino acid deficiencies on antibody formation in the rat. *J Nutr* **95**, 184-190.
Goldsby R, Kindt T, Osborne B, Kuby J (editors) (2003) *Immunology*, 5th ed., pp. 9-116. New York, USA: W H Freeman.
González-Ramón N, Alava MA, Sarsa JA, Piñeiro M, Escartin A, Garciagil A, Lampreave F, Piñeiro A (1995) The major acute phase serum protein in pigs is homologous to human plasma kallikrein sensitive PK-120. *FEBS Lett* **371**, 227-230.
González-Ramón N, Hoebe K, Alava MA, van Leengoed L, Piñeiro M, Carmona S, Iturralde M, Lampreave F, Piñeiro A (2000) PigMAP/ITIH4 and haptoglobin are interleukin-6-dependent acute-phase plasma proteins in porcine primary cultured hepatocytes. *Eur J Biochem* **267**, 1878-1885.
Greiner LL, Stahly TS, Stabel TJ (2000) Quantitative relationship of systemic virus concentration on growth and immune response in pigs. *J Anim Sci* **78**, 2690-2695.
Grimble RF (2001) Nutritional modulation of immune function. *Proc Nutr Soc* **60**, 389-397.
Grimble RF (2006) The effects of sulfur amino acid intake on immune function in humans. *J Nutr* **136**, 1660S-1665S.
Grimble RF, Grimble GK (1998) Immunonutrition: role of sulfur amino acids, related amino acids, and polyamines. *Nutr* **14**, 605-610.
Grimble RF, Jackson AA, Persaud C, Wride MJ, Delers F, Engler R (1992) Cysteine and glycine supplementation modulate the metabolic response to tumor necrosis factor a in rats fed a low protein diet. *J Nutr* **122**, 2066-2073.
Gruys E, Toussaint MJM, Niewold TA, Koopmans SJ (2005) Acute phase reaction and acute phase proteins. *J Zhejiang Univ Sci* **6B,**1045-1056.
Gutiérrez AM, Martínes-Subiela S, Soler L, Pallerés FJ, Cerón JJ (2009a) Use of saliva for haptoglobin and C-reactive protein quantifications in porcine respiratory and reproductive syndrome affected pigs in field conditions. *Vet Immunol Immunopathol* **132**, 218-223.

Gutiérrez AM, Martínez-Subiela S, Eckersall PD, Cerón JJ (2009b) C-reactive protein quantification in porcine saliva: A minimally invasive test for pig health monitoring. *Vet J* **181**, 261-265.
Gutteridge JMC (1987) The antioxidant activity of haptoglobin towards haemoglobin-stimulated lipid peroxidation. *Biochim Biophys Acta* **917**, 219-223.
Hale OM, Stewart TB, Marti OG (1985) Influence of an experimental infection of *Ascaris suum* on performance of pigs. *J Anim Sci* **60**, 220-225.
Hamard A, Sève B, Le Floc'h N (2007) Intestinal development and growth performance of early-weaned piglets fed a low-threonine diet. *Animal* **1**, 1134-1142.
Hasselgren PO (2000) Catabolic response to stress and injury: implications for regulation. *World J Surg* **24**, 1452-1459.
Hayaishi O (1996) Utilization of superoxide anion by indoleamine oxygenase-catalyzed tryptophan and indoleamine oxidation. *Adv Exp Med Biol* **398**, 285-289.
Heegaard P, Stockmarr A, Piñeiro M, Carpintero R, Lampreave F, Campbell F, Eckersall P, Toussaint M, Gruys, E, Sørensen N (2011) Optimal combinations of acute phase proteins for detecting infectious disease in pigs. *Vet Res* **42**, 50-63.
Heger J, Křížová L, Ŝustala M, Nitrayová S, Patráŝ P, Hampel D (2008) Individual response of growing pigs to sulphur amino acid intake. *J Anim Physiol Anim Nutr* **92**, 18-28.
Heger J, Křížová L, Ŝustala M, Nitrayová S, Patráŝ P, Hampel D (2009) Individual response of growing pigs to lysine intake. *J Anim Physiol Anim Nutr* **93**, 538-546.
Heinonen M (2001) Health classification of finnish swine herds - development measures and results. PhD Thesis, University of Helsinki, Finland.
Heinrich PC, Castell, J.V., and T Andus (1990) Interleukin-6 and the acute phase response. *Biochem J* **265**, 621-636.
Hill A, Brouwer A, Donaldson N, Lambton S, Buncic S, Griffiths I (2013) A risk and benefit assessment for visual-only meat inspection of indoor and outdoor pigs in the United Kingdom. *Food Control* **30**, 255-264.
Hoerr RA, Matthews DE, Bier DM, Young VR, (1993) Effects of protein restriction and acute refeeding on leucine and lysine kinetics in young men. *Am J Physiol Endocrinol Metab* **264**, 567-575.
Holck JT, Schinckel AP, Coleman JL, Wilt VM, Senn MK, Thacker BJ, Thacker EL, Grant AL (1998) The influence of environment on the growth of commercial finisher pigs. *Swine Health Prod* **6**, 141-149.
Holtrop G, Lapierre H, Lobley GE (2004) Modelling transport of amino acids into the red blood cells of sheep. *J Agr Sci* **142**, 577-588.
Hossein Sadrzadeh SM, Bozorgmehr J (2004) Haptoglobin phenotypes in health and disorders. *Am J Clin Pathol* **121**, 97S-104S.
Houdijk JGM, Campbell FM, Fortomaris PD, Eckersall PD, Kyriazakis I (2007) Effects of sub-clinical post-weaning colibacillosis and dietary protein on acute phase proteins in weaner pigs. *Livest Sci* **108**, 182-185.
Houdijk JGM, Jessop NS, Kyriazakis I (2001) Nutrient partitioning between reproductive and immune functions in animals. *Proc Nutr Soc* **60**, 515-525.
Houseman RA, Mcdonald I, Pennie K (1973) The measurement of total body water in living pigs by deuterium oxide dilution and its relation to body composition. *Br J Nutr* **30**, 149-156.
Huang L, Hogewind-Schoonenboom JE, de Groof F, Twisk JWR, Voortman GJ, Dorst K, Schierbeek H, Boehm G, Huang Y, Chen C, van Goudoever JB (2011) Lysine requirement of the enterally fed term infant in the first month of life. *Am J Clin Nutr* **94**, 1496-1503.
Hultén C, Johansson E, Fossum C, Wallgren P (2003) Interleukin 6, serum amyloid A and haptoglobin as markers of treatment efficacy in pigs experimentally infected with Actinobacillus pleuropneumoniae. *Vet Microbiol* **95**, 75-89.
Humphrey BD, D'Amato J, Moulds M (2008) Is immune function being traded off for optimal performance? In *Formula for the future: Nutrition or pathology? Elevating performance and health in pigs and poultry*, 1st ed., pp.139-157. Wageningen, The netherlands: Wageningen Academic Publishers.

Ijichi C, Matsumura T, Tsuji T, Eto Y (2003) Branched-chain amino acids promote albumin synthesis in rat primary hepatocytes through the mTOR signal transduction system. *Biochem Biophys Res Commun* **303**, 59-64.
International Organization for Standardization 6496 (1999) *Animal feeding stuff - Determination of moisture and other volatile matter content.* Geneva: ISO.
International Organization for Standardization 5983 (2005a). Animal feeding stuff - *Determination of nitrogen content and calculation of crude protein content - Part 1 Kjeldahl method.* Geneva: ISO.
International Organization for Standardization 13903 (2005b) *Animal feeding stuff - Determination of amino acids content*. Geneva: ISO.
International Organization for Standardization 13904 (2005c) *Animal feeding stuff - Determination of tryptophan content.* Geneva: ISO.
Iseri VJ, Klasing KC (2013) Dynamics of the systemic components of the chicken (*Gallus gallus domesticus*) immune system following activation by *Escherichia coli*: implications for the costs of immunity. *Dev Comp Immunol* **40**, 248-257.
Jackson AA, Phillips G, McClelland I, Jahoor F (2001) Synthesis of hepatic secretory proteins in normal adults consuming a diet marginally adequate in protein. *Am J Physiol Gastrointest Liver Physiol* **281**, 1179G -1187G.
Jahoor F, Wykes L, Del Rosario M, Frazer M, Reeds PJ (1999) Chronic protein undernutrition and an acute inflammatory stimulus elicit different protein kinetic responses in plasma but not in muscle of piglets. *J Nutr* **129**, 693-699.
Jahoor F, Wykes LJ, Reeds PJ, Henry JF, del Rosario MP, Frazer ME (1995) Protein-deficient pigs cannot maintain reduced glutathione homeostasis when subjected to the stress of inflammation. *J Nutr* **125**, 1462-1472.
Johnson RW (1998) Immune and endocrine regulation of food intake in sick animals. *Domest Anim Endocrin* **15**, 309-319.
Johnson RW (2002) The concept of sickness behavior: a brief chronological account of four key discoveries. *Vet Immunol Immunopathol* **87**, 443-450.
Kampman - van de Hoek E, Gerrits WJJ, van den Borne JJGC, van der Peet-Schwering CMC, van Beers-Schreurs H, Jansman AJM (2013) Challenge models to study the effect of immune system activation on amino acid metabolism in pigs. In *Proceedings of the 4th international symposium on energy and protein metabolism and nutrition*, 9-12 Sep 2013, Sacramento, USA, **134**, 237-238.
Kampman - van de Hoek E, Sakkas P, Gerrits WJJ, van den Borne JJGC, van der Peet-Schwering CMC, Jansman AJM (2015) Induced lung inflammation and dietary protein supply affect N retention and amino acid metabolism in growing pigs. *Br J Nutr* **113**, 414-425.
Kenney MA, Magee JL, Piedad-Pascual F (1970) Dietary amino acids and immune response in rats. *J Nutr* **100**, 1063-1072.
Kim CJ, Kovacs-Nolan JA, Yang C, Archbold T, Fan MZ, Mine Y (2010) l-Tryptophan exhibits therapeutic function in a porcine model of dextran sodium sulfate (DSS)-induced colitis. *J Nutr Biochem* **21**, 468-475.
Kim JC, Payne H, Collins C, Henman D, Mullan B (2012) Sulphur amino acid supplementation to imrove herd feed conversion efficiency in commercial grower production system. Report for the Co-operative Research Centre for High Integrity Australian Pork, South Perth, Australia.
Kim Kl, McMillan I, Bayley HS (1983) Determination of amino acid requirements of young pigs using an indicator amino acid. *Br J Nutr* **50**, 369-382.
Kipper M, Andretta I, Monteiro SG, Lovatto PA, Lehnen CR (2011) Meta-analysis of the effects of endoparasites on pig performance. *Vet Parasitol* **181**, 316-320.
Klasing KC (1988) Nutritional aspects of leukocytic cytokines. *J Nutr* **118**, 1436-1446.
Klasing KC, Barnes DM (1988) Decreased amino acid requirements of growing chicks due to immunologic stress. *J Nutr* **118**, 1158-1164.
Klasing KC (2007) Nutrition and the immune system. *Br Poult Sci* **48**, 525-537.

Klasing KC, Calvert CC (1999) The care and feeding of an immune system: an analysis of lysine needs. In *Proceedings of the 8th international symposium on protein metabolism and nutrition,* pp. 235-263. Wageningen, The Netherlands: Wageningen Press.
Klasing KC, Johnstone BJ (1991) Monokines in growth and development. *Poult Sci* **70**, 1781-1789.
Klasing K (2013) Recent advances in understanding the interactions between nutrients and immunity in farm animals. In *Proceedings of the 4th international symposium on energy and protein metabolism and nutrition*, 9-12 Sep 2013, Sacramento, USA, **134***,* 353-354.
Klem TB, Bleken E, Morberg H, Thoresen SI, Framstad T (2010) Hematologic and biochemical reference intervals for Norwegian crossbreed grower pigs. *Vet Clin Pathol* **39**, 221-226.
Kluger MJ (1991) Fever: Role of pyrogens and cryogens. *Physiol Rev* **71**, 101-127.
Knura-Deszczka S, Lipperheide C, Petersen B, Jobert JL, Berthelot-Hérault F, Kobisch M, Madec F (2002) Plasma haptoglobin concentration in swine after challenge with *Streptococcus suis*. *J Vet Med* **49**, 240-244.
Konashi S, Takahashi K, Akiba Y (2000) Effects of dietary essential amino acid deficiencies on immunological variables in broiler chickens. *Br J Nutr* **83**, 449-456.
Koopmans SJ, van der Meulen J, Dekker R, Corbijn H, Mroz Z (2005) Diurnal rhythms in plasma cortisol, insulin, glucose, lactate and urea in pigs fed identical meals at 12-hourly intervals. *Physiol Behav* **84**, 497-503.
Koops WJ, Grossman M (1993) Multiphasic allometry. *Growth Develop Aging* **57**, 183-192.
Kurpad AV, Jahoor F, Borgonha S, Poulo S, Rekha S, Fjeld CR, Reeds PJ (1999) A minimally invasive tracer protocol is effective for assessing the response of leucine kinetics and oxidation to vaccination in chronically energy-deficient adult males and children. *J Nutr* **129**, 1537-1544.
Kushner I, Rzewnicki DL (1994) The acute phase response: General aspects. *Baillière Clin Rheum* **8**, 513-530.
Kyriazakis I, Emmans GC (1992) The growth of mammals following a period of nutritional limitation. *J Theor Biol* **156**, 485-498.
Kyriazakis I, Emmans GC, McDaniel R (1993) Whole body amino acid composition of the growing pig. *J Sci Food Agr* **62**, 29-33.
Kyriazakis I, Houdijk J (2006) Immunonutrition: Nutritional control of parasites. *Small Ruminant Res* **62**, 79-82.
Kyriazakis I, Doeschl-Wilson A (2009) Anorexia during infection in mammals: variation and its sources. In *Voluntary food intake in pigs*, 1st ed., pp. 307-322. Wageningen, the Netherlands: Wageningen Academic Publishers.
Lampreave F, González-Ramón N, Martínez-Ayensa S, Hernández MA, Lorenzo HK, García-Gil A, Piñeiro A (1994) Characterization of the acute phase serum protein response in pigs. *Electrophoresis* **15**, 672-676.
Le Floc'h N, Melchior D, Obled C (2004) Modifications of protein and amino acid metabolism during inflammation and immune system activation. *Livest Prod Sci* **87**, 37-45.
Le Floc'h N, Jondreville C, Matte JJ, Sève B (2006) Importance of sanitary environment for growth performance and plasma nutrient homeostasis during the post-weaning period in piglets. *Arch Anim Nutr* **60**, 23-34.
Le Floc'h N, Melchior D, Sève B (2008) Dietary tryptophan helps to preserve tryptophan homeostasis in pigs suffering from lung inflammation. *J Anim Sci* **86**, 3473-3479.
Le Floc'h N, Lebellego L, Matte JJ, Melchior D, Sève B (2009) The effect of sanitary status degradation and dietary tryptophan content on growth rate and tryptophan metabolism in weaning pigs. *J Anim Sci* **87**, 1686-1694.
Le Floc'h N, Matte JJ, Melchior D, van Milgen J, Sève B (2010) A moderate inflammation caused by the deterioration of housing conditions modifies Trp metabolism but not Trp requirement for growth of post-weaned piglets. *Animal* **4**, 1891-1898.
Lecker SH, Solomon V, Mitch WE, Goldberg AL (1999) Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *J Nutr* **129**, 227S-237S.

Lee HY, Kim SD, Baek S-H, Choi JH, Cho K-H, Zabel BA, Bae Y-S (2013) Serum amyloid A stimulates macrophage foam cell formation via lectin-like oxidized low-density lipoprotein receptor 1 upregulation. *Biochem Biophys Res Commun* **433**, 18-23.
Leneveu P, Robert N, Keita A, Pagot E, Pommier P, Teissier P (2005) Lung lesions in pigs at slaughter: a 2-year epidemiological study in France. *Int J Appl Res Vet M* **3**, 259-265.
Lewis B, Langkamp-Henken B (2000) Arginine enhances *in vivo* immune responses in young, adult and aged mice. *J Nutr* **130**, 1827-1830.
Li DF, Xiao CT, Qiao SY, Zhang JH, Johnson EW, Thacker PA (1999) Effects of dietary threonine on performance, plasma parameters and immune function of growing pigs. *Anim Feed Sci Tech* **78**, 179-188.
Li P, Yin Y-L, Li D, Kim SW, Wu G (2007) Amino acids and immune function. *Br J Nutr* **98**, 237-252.
Lipperheide C, Diepers N, Lampreave F, Alava M, Petersen B (1998) Nephelometric determination of haptoglobin plasma concentrations in fattening pigs. *J Vet Med A* **45**, 543-550.
Litvak N, Htoo JK, de Lange CFM (2013a) Restricting sulfur amino acid intake in growing pigs challenged with lipopolysaccharides decreases plasma protein and albumin synthesis. *Can J Anim Sci* **93**, 505-515.
Litvak N, Rakhshandeh A, Htoo JK, de Lange CFM (2013b) Immune system stimulation increases the optimal dietary methionine to methionine plus cysteine ratio in growing pigs. *J Anim Sci* **91**, 4188-4196.
Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**, 87-98.
Lorin J, Zeller M, Guilland J-C, Cottin Y, Vergely C, Rochette L (2014) Arginine and nitric oxide synthase: Regulatory mechanisms and cardiovascular aspects. *Mol Nutr Food Res* **58**, 101-116.
Lu SC (2009) Regulation of glutathione synthesis. *Mol Aspects Med* **30**, 42-59.
Mackiewicz A, Kwang WJ (1997) Acute phase proteins and transformed cells. In *International review of cytology*, vol. 170, pp. 225-300 [KW Jeon, editor]. London, UK: Academic Press.
Maclachlan NJ, Dubovi EJ (editors) (2011) Epidemiology and control of viral diseases. In *Fenner's veterinary virology*, 4th ed., pp. 136. London, UK: Academic Press.
Macmicking J, Xie QW, Nathan C (1997) Nitric oxide and macrophage function. *Ann Rev Immunol* **15**, 323-350.
MacRae JC (1993) Metabolic consequences of intestinal parasitism. *Proc Nutr Soc* **52**, 121-130.
Maes M, Meltzer HY, Scharpè S, Bosmans E, Suy E, De Meester I, Calabrese J, Cosyns P (1993) Relationships between lower plasma L-tryptophan levels and immune-inflammatory variables in depression. *Psychiatr Res* **49**, 151-165.
Mair KH, Sedlak C, Käser T, Pasternak A, Levast B, Gerner W, Saalmüller A, Summerfield A, Gerdts V, Wilson HL, Meurens F (2014) The porcine innate immune system: An update. *Dev Comp Immunol* **45**, 321-343.
Malmezat T, Breuillé D, Pouyet C, Mirand PP, Obled C (1998) Metabolism of cysteine is modified during the acute phase of sepsis in rats. *J Nutr* **128**, 97-105.
Malmezat T, Breuillé D, Capitan P, Mirand PP, Obled C (2000) Glutathione turnover is increased during the acute phase of sepsis in rats. *J Nutr* **130**, 1239-1246.
Mandi Y, Vécsei L (2012) The kynurenine system and immunoregulation. *J Neural Transm* **119**, 197-209.
Matthews DE, Downey RS (1984) Measurement of urea kinetics in humans: a validation of stable isotope tracer methods. *Am J Physiol Endocrinol Metab* **246**, 519E-527E.
Melchior D, Mézière N, Sève B, Le Floc'h N (2005) Is tryptophan catabolism increased under indoleamine 2,3 dioxygenase activity during chronic lung inflammation in pigs? *Reprod Nutr Dev* **45**, 175-183.
Melchior D, Sève B, Le Floc'h N (2004) Chronic lung inflammation affects plasma amino acid concentrations in pigs. *J Anim Sci* **82**, 1091-1099.
Mellor AL, Munn DH (2004) Ido expression by dendritic cells: tolerance and tryptophan catabolism. *Nature Rev Immunol* **4**, 762-774.

Merialdi G, Dottori M, Bonilauri P, Luppi A, Gozio S, Pozzi P, Spaggiari B, Martelli P (2012) Survey of pleuritis and pulmonary lesions in pigs at abattoir with a focus on the extent of the condition and herd risk factors. *Vet J* **193**, 234-239.
Merlot E, Vincent A, Thomas F, Meunier-Salaün M-C, Damon M, Robert F, Dourmad J-Y, Lebret B, Prunier A (2012) Health and immune traits of Basque and Large White pigs housed in a conventional or enriched environment. *Animal* **6**, 1290-1299.
Meyns T, van Steelant J, Rolly E, Dewulf J, Haesebrouck F, Mae, D (2011) A cross-sectional study of risk factors associated with pulmonary lesions in pigs at slaughter. *Vet J* **187**, 388-392.
Moehn S, Shoveller AK, Rademacher M, Ball RO (2008) An estimate of the methionine requirement and its variability in growing pigs using the indicator amino acid oxidation technique. *J Anim Sci* **86**, 364-369.
Moffett JR, Namboodiri MAA (2003) Tryptophan and the immune response. *Immunol Cell Biol* **81**, 247-265.
Montagne L, Lovatto PA, van Milgen J (2010) Effects of different sanitary challenges on voluntary feed intake in growing pigs. *Livest Sci* **134**, 252-254.
Moraes ML, Ribeiro AML, Kessler AM, Ledur VS, Fischer MM, Bockor L, Cibulski SP, Gava D (2012) Effect of conjugated linoleic acid on performance and immune response of weanling piglets. *J Anim Sci* **90**, 2590-2598.
Mousing J, Lybye H, Barfod K, Meyling A, Rønsholt L, Willeberg P (1990) Chronic pleuritis in pigs for slaughter: an epidemiological study of infectious and rearing system-related risk factors. *Prev Vet Med* **9**, 107-119.
Murata H, Shimada N, Yoshioka M (2004) Current research on acute phase proteins in veterinary diagnosis: an overview. *Vet J* **168**, 28-40.
Nederlands Normalisatie Instituut (1992) NEN 3574. NNI-catalogus I. Delft: NNI.
Netea MG, Kullberg BJ, Van der Meer JWM (2000) Circulating cytokines as mediators of fever. *Clin Infect Dis* **31**, 178S-184S.
Newsholme P (2001) Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection? *J Nutr* **131**, 2515-2522.
Nishimura H, Kakizaki I, Muta T, Sasaki N, Pu PX, Yamashita T, Nagasawa S (1995) cDNA and deduced amino acid sequence of human PK-120, a plasma kallikrein-sensitive glycoprotein. *FEBS Lett* **357**, 207-211.
NRC (1998) *Nutrient Requirements of Swine*. 10t^{h} ed., Washington, USA: National Academy Press.
NRC (2012) *Nutrient Requirements of Swine*. 11th ed., Washington, USA: National Academy Press.
Obled C (2003) Amino acid requirements in inflammatory states. *Can J Anim Sci* **83**, 365-373.
Oosterveld MJS, Reinoud JBJG, Jack RD, Willem K, Cornelis J, Kees de M (2005) Minimal sampling protocol for accurate estimation of urea production: a study with oral [^{13}C]urea in fed and fasted piglets. *Clin Nutr* **24**, 97-104.
Paajanen (2005) Effects of cow's milk and its processing on gastrointestinal symptoms and delayed-type immune responses. PhD Thesis, University of Helsinki, Finland.
Pabst R, Binns RM (1994) The immune system of the respiratory tract in pigs. *Vet Immunol Immunopathol* **43**, 151-156.
Pallarés FJ, Martínez-Subiela S, Seva J, Ramis G, Fuentes P, Bernabé A, Muñoz A, Cerón JJ (2008) Relationship between serum acute phase protein concentrations and lesions in finishing pigs. *Vet J* **177**, 369-373.
Parkin J, Cohen B (2001) An overview of the immune system. *Lancet* **357**, 1777-1789.
Parra MD, Fuentes P, Tecles F, Martínez-Subiela S, Martínez JS, Muñoz A, Cerón JJ (2006) Porcine acute phase protein concentrations in different diseases in field conditions. *J Vet Med B* **53**, 488-493.
Pastorelli H, van Milgen J, Lovatto P, Montagne L (2012) Meta-analysis of feed intake and growth responses of growing pigs after a sanitary challenge. *Animal* **6**, 952-961.
Pencharz PB, Ball RO (2003) Different approaches to define individual amino acid requirement. *Ann Rev Nutr* **23**, 101-116.

Petersen HH, Dideriksen D, Christiansen BM, Nielsen JP (2002a) Serum haptoglobin concentration as a marker of clicinal signs in finishing pigs. *Vet Rec* **151**, 85-89.
Petersen HH, Ersbøll AK, Jensen CS, Nielsen JP (2002) Serum-haptoglobin concentration in Danish slaughter pigs of different health status. *Prev Vet Med* **54**, 325-335.
Petersen HH, Nielsen JP, Heegaard PM (2004) Application of acute phase protein measurements in veterinary clinical chemistry. *Vet Res* **35**, 163-187.
Piñeiro M, Morales J, Vizcaíno E, Murillo JA, Klauke T, Petersen B, Piñeiro C (2013) The use of acute phase proteins for monitoring animal health and welfare in the pig production chain: The validation of an immunochromatographic method for the detection of elevated levels of pig-MAP. *Meat Sci* **95**, 712-718.
Popovic PJ, Zeh HJ, Ochoa JB (2007) Arginine and immunity. *J Nutr* **137**, 1681S-1686.
PVV (2006) *Handboek registratie IKB afwijkingen vleesvarkens*. Zoetermeer, The Netherlands: Product Boards for Livestock and Meat.
Qiu S, Fang Z, Wu D, Lin Y, Che L (2011) Tryptophan supplements promote pregnancy success in mice challenged with Pseudorabies Virus (PRV) by regulating the expression of systemic cytokines, immunoglobulins, PRV-specific protein profiles, and Toll-like receptors. *J Medicin Food* **14**, 857-865.
Rakhshandeh A, Htoo JK, de Lange CFM (2010) Immune system stimulation of growing pigs does not alter apparent ileal amino acid digestibility but reduces the ratio between whole body nitrogen and sulfur retention. *Livest Sci* **134**, 21-23.
Rakhshandeh A, Htoo JK, Karrow N, Miller SP, de Lange CFM (2014) Impact of immune system stimulation on ileal nutrient digestibility and utilization of methionine plus cysteine intake for whole body protein deposition in growing pigs. *Br J Nutr* **111**, 101-110.
Rao DS, McCracken KJ (1990) Protein requirements of boars of high genetic potential for lean growth. *Anim Prod* **51**, 179-187.
Raz A, Goodman DS (1969) The interaction of thyroxine with human plasma prealbumin and with the prealbumin-retinol-binding protein complex. *J Biol Chem* **244**, 3230-3237.
Reeds PJ, Cadenhead A, Fuller MF, Lobley GE, McDonald JD (1980) Protein turnover in growing pigs. Effects of age and food intake. *Br J Nutr* **43**, 445-455.
Reeds PJ, Fuller MF, Cadenhead A, Hay SM (1987) Urea synthesis and leucine turnover in growing pigs: changes during 2 d following the addition of carbohydrate or fat to the diet. *Br J Nutr* **58**, 301-311.
Reeds PJ, Fjeld CR, Jahoor F (1994) Do the differences between the amino acid compositions of acute-phase and muscle proteins have a bearing on nitrogen loss in traumatic states? *J Nutr* **124**, 906-910.
Reeds PJ, Jahoor F (2001) The amino acid requirements of disease. *Clin Nutr* **20**, 15-22.
Reeves DE (2006) The application of biotechnical and epidemiologic tools for pig health. *Anim Biotechnol* **17**, 177-187.
Renaudeau D (2009) Effect of housing conditions (clean *vs*. dirty) on growth performance and feeding behavior in growing pigs in a tropical climate. *Trop Anim Health Pro* **41**, 559-563.
Rivera-Ferre MG, Aguilera JF, Nieto R (2006) Differences in whole-body protein turnover between Iberian and Landrace pigs fed adequate or lysine-deficient diets. *J Anim Sci* **84**, 3346-3355.
Roth E (2007) Immune and cell modulation by amino acids. *Clin Nutr* **26**, 535-544.
Roth E, Oehler r, Manhart N, Exner R, Wessner B, Strasser E, Spittler A (2002) Regulative potential of glutamine-relation to glutathione metabolism. *Nutr* **18**, 217-221.
Rothschild MA, Oratz M, Schreiber SS (1969) Serum albumin. *New Series* **14**, 711-744.
Ruth MR, Field CJ (2013) The immune modifying effects of amino acids on gut-associated lymphoid tissue. *J Anim Sci Biotech* **4**, 27-37.
Sakkas P, Jones LA, Houdijk JGM, Athanasiadou S, Knox DP, Kyriazakis I (2012) Leucine and methionine deficiency impairs immunity to gastrointestinal parasites during lactation. *Br J Nutr* **28**, 273-282.
Salamano G, Mellia E, Candiani D, Ingravalle F, Bruno R, Ru G, Doglione L (2008) Changes in haptoglobin, C-reactive protein and pig-MAP during a housing period following long distance transport in swine. *Vet J* **177**, 110-115.

Salter DN, Montgomery AI, Hudson A, Quelch DB, Elliott RJ (1990) Lysine requirements and whole-body protein turnover in growing pigs. *Br J Nutr* **63**, 503-513.
Sandberg FB, Emmans GC, Kyriazakis I (2006) A model for predicting feed intake of growing animals during exposure to pathogens. *J Anim Sci* **84**, 1552-1566.
Sandberg FB, Emmans GC, Kyriazakis I (2007) The effects of pathogen challenges on the performance of naïve and immune animals: the problem of prediction. *Animal* **1**, 67-86.
Sauvant D, Perez JM, Tran G (editors) (2004) *Tables de composition et de valeur nutritive des matières premières destinées aux animaux d'élevage Porcs, volailles, bovins, ovins, caprins, lapins, chevaux, poissons*. 2nd ed. Paris, France: INRA.
Schakman O, Dehoux M, Bouchuari S, Delaere S, Lause P, Decroly N, Shoelson SE, Thissen JP (2012) Role of IGF-I and the TNF alpha/NF-kappa B pathway in the induction of muscle atrogenes by acute inflammation. *Am J Physiol Endocrinol Metab* **303**, 729E-739E.
Schleicher C, Scheriau S, Kopacka I, Wanda S, Hofrichter J, Köfer J (2013) Analysis of the variation in meat inspection of pigs using variance partitioning. *Prev Vet Med* **111**, 278-285.
Schreiber G, Richardson SJ (1997) The evolution of gene expression, structure and function of transthyretin. *Comp Biochem Physiol B Biochem Mol Biol* **116**, 137-160.
Skovgaard K, Mortensen S, Boye M, Poulsen KT, Campbell FM, Eckersall PD, Heegaard PMH (2009) Rapid and widely disseminated acute phase protein response after experimental bacterial infection of pigs. *Vet Res* **40**, 23-35.
Song C, Hsu K, Yamen E, Yan W, Fock J, Witting PK, Geczy CL, Freedman SB (2009) Serum amyloid A induction of cytokines in monocytes/macrophages and lymphocytes. *Atherosclerosis* **207**, 374-383.
Sørensen NS, Tegtmeier C, Andresen LO, Piñeiro M, Toussaint MJM, Campbell FM, Lampreave F, Heegaard PMH (2006) The porcine acute phase protein response to acute clinical and subclinical experimental infection with *Streptococcus suis*. *Vet Immunol Immunopathol* **113**, 157-168.
Sørensen V, Jorsal SE, Mousing J (2006) Diseases of the respiratory system. In *Diseases of swine*, 9th ed., pp. 149-178. Ames, USA: Blackwell Publishing.
Spurlock ME (1997) Regulation of metabolism and growth during immune challenge: an overview of cytokine function. *J Anim Sci* **75**, 1773-1783.
Spurlock ME, Frank GR, Willis GM, Kuske JL, Cornelius SG (1997) Effect of dietary energy source and immunological challenge on growth performance and immunological variables in growing pigs. *J Anim Sci* **75**, 720-726.
Stark KDC (2000) Epidemiological investigation of the influence of environmental risk factors on respiratory diseases in swine - A literature review. *Vet J* **159**, 37-56.
Stechmiller JK, Childress B, Cowan L (2005) Arginine supplementation and wound healing. *Nutr Clin Pract* **20**, 52-61.
Suffredini AF, Fantuzzi G, Badolato R, Oppenheim JJ, O'Grady NP (1999) New insights into the biology of the acute phase response. *J Clin Immunol* **19**, 203-214.
Susenbeth A (1995) Factors affecting lysine utilization in growing pigs: an analysis of literature data. *Livest Prod Sci* **43**, 193-204.
Susenbeth A, Schneider R, Menke KH (1994) The effect of protein and lysine intake on growth and protein retention in pigs. *J Anim Physiol Anim Nutr* **71**, 200-207.
Sutherland MA, Rodriguez-Zas SL, Ellis M, Salak-Johnson JL (2005) Breed and age affect baseline immune traits, cortisol, and performance in growing pigs. *J Anim Sci* **83**, 2087-2095.
Sutherland MA, Niekamp SR, Rodriguez-Zas SL, Salak-Johnson JL (2006) Impacts of chronic stress and social status on various physiological and performance measures in pigs of different breeds. *J Anim Sci* **84**, 588-596.
Temple D, Dalmau A, Ruiz de la Torre JL, Manteca X, Velarde A (2011) Application of the Welfare Quality® protocol to assess growing pigs kept under intensive conditions in Spain. *J Vet Behav* **6**, 138-149.

Toussaint MJM, Ederen AM, Gruys E (1995) Implication of clinical pathology in assessment of animal health and in animal production and meat inspection. *Comp Haematol Int* **5**, 149-157.
Tullis JB, Whittemore CT, Phillips P (1986) Compensatory nitrogen retention in growing pigs following a period of N deprivation. *Br J Nutr* **56**, 259-267.
Underhill DM, Ozinsky A (2002) Phagocytosis of microbes: Complexity in action. *Ann Rev Immunol* **20**, 825-252.
Urynek W, Buraczewska L (2003) Effect of dietary energy concentration and apparent ileal digestible lysine:metabolizable energy ratio on nitrogen balance and growth performance of young pigs. *J Anim Sci* **81**, 1227-1236.
Van der Peet-Schwering CMC, Jansman AJM (2007) *Nutriëntbehoefte van varkens onder suboptimale omstandigheden*. NL Rapport no. 68. Lelystad, The Netherlands: WUR Livestock Research.
Van der Peet-Schwering CMC, Straathof SB, Binnendijk GP, van Diepen JTM (2012) *Influence of diet composition and level of amino acids on performance of boars, barrows and gilts*. Report 563. Lelystad, The Netherlands: WUR Livestock Research.
Van Heugten E, Coffey MT, Spears JW (1996) Effects of immune challenge, dietary energy density, and source of energy on performance and immunity in weanling pigs. *J Anim Sci* **74**, 2431-2440.
Van Kleef DJ, Deuring K, van Leeuwen P (1994) A new method of faeces collection in the pig. *Lab Anim* **28**, 78-79.
van Milgen J, Valancogne A, Dubois S, Dourmad JY, Sève B, Noblet J (2008) InraPorc: A model and decision support tool for the nutrition of growing pigs. *Anim Feed Sci Technol* **143**, 387-405.
Van Wagenberg CPA, Backus GBC, Kuiper WE, van der Vorst JGAJ, Urlings HAP (2010) Incentive mechanisms for liver lesion control in finishing pigs in the Netherlands. *Prev Vet Med* **93**, 19-24.
Vicario M, Amat C, Rivero M, Moreto M, Pelegri C (2007) Dietary glutamine affects mucosal functions in rats with mild DSS-induced colitis. *J Nutr* **137**, 1931-1937.
Vigushin DM, Pepys MB, Hawkins PN (1993) Metabolic and scintigraphic studies of radioiodinated human C-reactive protein in health and disease. *J Clin Invest* **91**, 1351-1357.
Vlaminck J, Levecke B, Vercruysse J, Geldhof P (2014) Advances in the diagnosis of *Ascaris suum* infections in pigs and their possible applications in humans. *Parasitol* **141**, 1-8.
Wang WW, Qiao SY, Li DF (2009) Amino acids and gut function. *Amino Acids* **37**, 105-110.
Wang X, Qiao SY, Liu M, Ma YX (2006a) Effects of graded levels of true ileal digestible threonine on performance, serum parameters and immune function of 10-25 kg pigs. *Anim Feed Sci Tech* **129**, 264-278.
Wang X, Qiao SY, Liu M, Ma YX (2006b) Effects of graded levels of true ileal digestible threonine on performance, serum parameters and immune function of 10-25 kg pigs. *Anim Feed Sci Tech* **129**, 264-278.
Waterlow JC (editor) (2006) *Protein turnover*, 1st ed., pp. 87-102. Cambridge, UK: CAB International.
Waterlow JC, Golden MH, Garlick PJ (1978) Protein turnover in man measured with ^{15}N: comparison of end products and dose regimes. *Am J Physiol Endocrinol Metab* **235**, 165E-174E.
Webel DM, Finck BN, Baker DH, Johnson RW (1997) Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. *J Anim Sci* **75**, 1514-1520.
Webel DM, Johnson RW, Baker DH (1998) Lipopolysaccharide-induced reductions in food intake do not decrease the efficiency of lysine and threonine utilization for protein accretion in chickens. *J Nutr* **128**, 1760-1766.
Wei R, Zimmerman DR (2003) An evaluation of the NRC (1998) growth model in estimating lysine requirements of barrows with a lean growth rate of 348 g/d. *J Anim Sci* **81**, 1772-1780.

Wheeler MD, Rose ML, Yamashima S, Enomoto N, Seabra V, Madren J, Thurman RG (2000) Dietary glycine blunts lung inflammatory cell influx following acute endotoxin. *Am J Physiol-Lung Cell Mol Physiol* **279**, 390L-398L.
Willeberg P, Gerbola MA, Petersen BK, Andersen JB (1984) The Danish pig health scheme - Nation-wide computer-based abattoir surveillance and follow-up at the herd level. *Prev Vet Med* **3**, 79-91.
Williams NH, Stahly TS, Zimmerman DR (1997a) Effect of chronic immune system activation on the rate, efficiency, and composition of growth and lysine needs of pigs fed from 6 to 27 kg. *J Anim Sci* **75**, 2463-2471.
Williams NH, Stahly TS, Zimmerman DR (1997b) Effect of level of chronic immune system activation on the growth and dietary lysine needs of pigs fed from 6 to 112 kg. *J Anim Sci* **75**, 2481-2496.
Williams NH, Stahly TS, Zimmerman DR (1997c) Effect of chronic immune system activation on body nitrogen retention, partial efficiency of lysine utilization, and lysine needs of pigs. *J Anim Sci* **75**, 2472-2480.
Wilson MR, Takov R, Friendship RM, Martin SW, Mcmillan I, Hacker RR, Swaminathan S (1986) Prevalence of respiratory diseases and their association with growth rate and space in randomly selected swine herds. *Can J Vet Res* **50**, 209-216.
Wing K, Sakaguchi S (2010) Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nature Immunol* **11**, 7-13.
Winters KA, Mathes LE, Krakowka S, Olsen RG (1983) Immunoglobulin class response to canine distemper virus in gnotobiotic dogs. *Vet Immunol Immunopathol* **5**, 209-215.
Wolfe RR (1999) Alterations in protein metabolism due to the stress of injury and infection. In *The role of protein and amino acids in sustaining and enhancing performance*, 1st ed., pp. 279-284. Washington, USA: National Academy Press.
World Health Organisation (1948) *Preamble to the constitution of the World Health Organization.* Official Records of the World Health Organization, no. 2, pp. 100. Geneva, Switserland: WHO.
World organisation for animal health (OIE), 2011. Terrestrial animal health code. http://www.oie.int (accessed Jan 2014).
Wu D (2013) Functional amino acids in nutrition and health. *Amino Acids* **45**, 407-411.
Wu G, Meier SA, Knabe DA (1996) Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. *J Nutr* **126**, 2578-2584.
Wu G, Meininger CJ, Knabe DA, Bazer FW, Rhoads JM (2000) Arginine nutrition in development, health and disease. *Curr Opin Clin Nutr Metab Care* **3**, 59-66.
Wu G, Morris SM (1998) Arginine metabolism: nitric oxide and beyond. *Biochem J* **336**, 1-17.
Wu GY (2009) Amino acids: metabolism, functions, and nutrition. *Amino Acids* **37**, 1-17.
Young VR, El-Khoury AE, Raguso CA, Forslund AH, Hambraeus L (2000) Rates of urea production and hydrolysis and leucine oxidation change linearly over widely varying protein intakes in healthy adults. *J Nutr* **130**, 761-766.
Zamir O, Hasselgren PO, Higashiguchi T, Frederick JA, Fischer JA (1992) Tumour necrosis factor (TNF) and interleukin-1 (IL-1) induce muscle proteolysis through different mechanisms. *Mediators Inflamm* **1**, 247-250.
Zhang G, Ross CR, Dritz SS, Nietfeld JC, Blecha F (1997) Salmonella infection increases porcine antibacterial peptide concentrations in serum. *Clin Vaccine Immunol* **4**, 774-777.

Summary

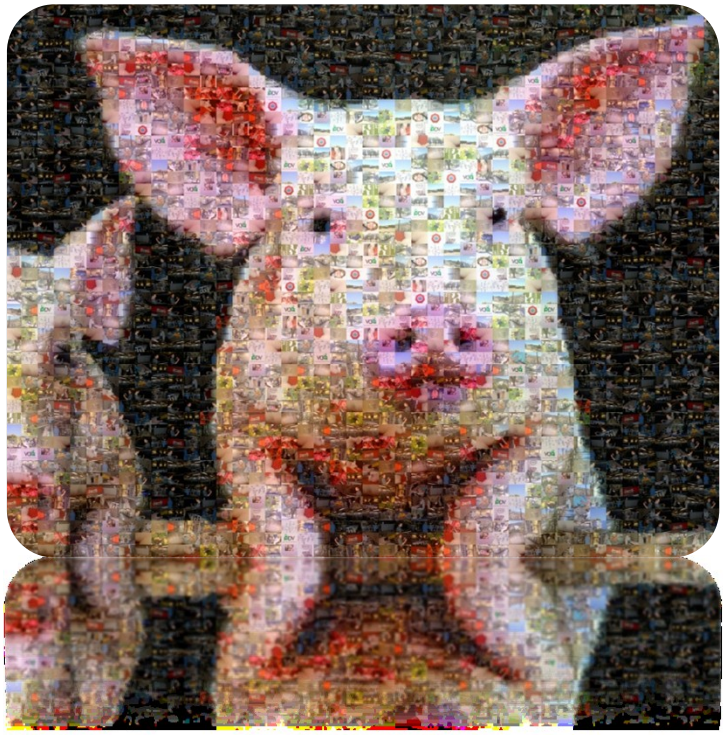
Samenvatting

Acknowledgements

About the author

(Curriculum Vitae, List of publications, Training and Supervision Plan)

List of abbreviations



Summary

There is large variation in the production performance of commercial growing-finishing pig farms. This variation even exists when pigs have a similar genetic background and fed similar diets. The variation in health status between growing-finishing pig farms is suggested to be one of the major factors contributing to this large variation in pig performance. In commercial farms, pigs can be continuously exposed to high levels of (non-) pathogenic agents that can activate the immune system, and in turn affect protein and amino acid (AA) metabolism. Quantitative information about the AA requirements of pigs with an activated immune system is limited. This information enables feed manufacturers to optimize pig diets by adjusting to variation in health status, and thereby further improving pig performance. However, for the implementation of targeted feeding strategies, it is pivotal to classify farms on the basis of their health status. In addition, experimental challenge models are required to study the effect of immune system activation on AA metabolism, that are relevant to the contrast in health status observed in commercial pig farms. In addition, techniques are required which quantify changes in AA metabolism. The main objective of the present thesis was to quantify the effect of health status on AA requirements for body protein deposition and for immune system functioning of growing pigs.

In the study described in **Chapter 2** a health status web was developed as a concept for the classification of growing-finishing pig farms, based on data recorded in current commercial practice. Six traits were incorporated into a health status web, being average daily gain, energy conversion ratio, mortality, incidence of pleuritis, and percentage of rejected lungs and livers at slaughter over a period of one year. Performance data from 1074 and 783 Dutch pig farms, and abattoir data of 50208 and 47426 farm deliveries to slaughterhouses, acquired over 2011 and 2012 respectively, were used as a representative sample for the Dutch growing-finishing pig population. For each individual farm, a score was calculated per trait by inter- and extrapolation using the data of the Dutch growing-finishing pig population as reference. The mean score over the six traits, was used to classify farms as having a suboptimal, conventional or high health status. The health status web can be of use for feed manufacturers to develop targeted strategies to accommodate the nutritional requirements of pigs belonging to particular groups of farms sharing a common health status, in turn improving efficiency of pig production.

In the studies described in **Chapter 3, 4 and 5** techniques were developed to determine AA requirements and to measure changes in AA metabolism. The study described in Chapter 3 indicated that the dose-response technique is a valuable method to determine AA requirements using a decreasing dietary lysine (Lys) supply strategy in time and a step length of 3 day with urinary nitrogen (N) excretion as response criterion. The dose-response technique is a simple, accurate technique to quantitatively estimate changes in AA requirements of individual meal-fed pigs. Nevertheless, a minimum time period of 21 days is required for each individual (a step

length of 3 days · at least 7 dietary AA levels), which makes the technique inappropriate for situations in which it is difficult to maintain a steady state *e.g.* in case of studying the effects of immune system activation on the AA requirement. In addition, dose-response studies typically estimate the requirement of a single AA, but do not provide insight in simultaneous changes in the utilization of other AA. Measurements on the plasma irreversible loss rate (ILR, *i.e.* the amount of free AA that disappears per unit of time from the plasma pool for protein synthesis or oxidation) can be performed for multiple AA simultaneously, allowing estimation of a shift in metabolism of different AA. The combined measurements of whole body N retention and rates of ILR of AA from plasma, urea entry and appearance of ^{13}C into plasma proteins (after a bolus of ^{13}C labelled AA and $^{15}N_2$ urea) provided insight into the consequences of immune system activation on AA metabolism.

In the study described in **Chapter 4** growing pigs of approximately 8 weeks old were obtained from a farm characterized as having a high health (n = 14) or low health (n = 14) status, as assessed by the presence of antibodies against pathogens and sanitary status. Pigs were allocated to a diet adequate in essential AA or deficient in methionine (Met) + cysteine (Cys), threonine (Thr) and tryptophan (Trp). Low health status pigs had greater serum haptoglobin, lower serum albumin concentrations, and greater leukocyte counts in blood than high health status pigs, indicating a higher level of immune system activation at the start of the experiment. Total tract dry matter and N digestibility was lower in low health status pigs than in high health status pigs. Low health status pigs on a diet adequate in essential AA showed, however, compensatory body weight gain upon arrival, coinciding with a greater N retention and greater efficiency of N utilization compared to high health status pigs. Low health status pigs showed a greater plasma ILR for Lys, and a greater urea pool size than pigs with a high health status, indicating greater oxidation of AA due to an imbalance in AA, especially in low health status pigs fed a diet deficient in essential AA. The results suggested that the competition for AA between synthesis of proteins associated with immune system activation and body protein deposition is enlarged when dietary supply of Met + Cys, Thr and Trp is restricted.

In the study described in **Chapter 5** a total of 16 barrows received an adequate or restricted amount of dietary protein, and were challenged with intravenous (*i.v.*) complete Freund's adjuvant (CFA) to induce a systemic lung inflammation. Serum acute phase proteins, N retention measurements, and the ILR of eight AA were determined pre- and post-challenge. CFA successfully activated the immune system, as indicated by a 2- to 4-fold increase in serum concentrations of APP. The CFA challenge increased urinary N losses and tended to reduce N retention in pigs fed an adequate amount of dietary protein, but not in pigs fed a restricted amount. The Met pool size was approximately 230% greater at day 8 post-challenge than at day 3 post-challenge in pigs fed an adequate amount of dietary protein. The ILR for valine (Val) was lower at day 8 than at day 3 in the post-challenge period. The observed changes in protein metabolism imply that especially Trp may become limiting during immune system activation, whereas Lys becomes excessive (**General Discussion**). Furthermore, the

utilization of Met, tyrosine (Tyr), and Val for immune system functioning seems to increase in pigs with a systemic lung inflammation.

A dietary amino acid or protein supply can modulate the acute phase response pre- and post-challenge, stressing the importance of an adequate dietary amino acid supply for appropriate functioning of the immune system of growing-finishing pigs (Chapter 4 and 5).

Before implementing targeted feeding strategies for farms sharing a common health status, future research should be conducted to study the possible beneficial effects of increasing the dietary supply of particularly Trp, Met, Tyr, and Val relative to Lys for immune system function and for body protein deposition in pigs from farms with a suboptimal health status.

Samenvatting

Er is een grote variatie in technische resultaten van commerciële vleesvarkensbedrijven. Deze variatie bestaat zelfs wanneer varkens eenzelfde genotype hebben en hetzelfde voer verstrekt krijgen. De variatie in gezondheidsstatus tussen vleesvarkensbedrijven is waarschijnlijk een van de belangrijkste factoren die bijdraagt aan de grote variatie in technische resultaten. Op commerciële bedrijven kunnen varkens continu blootgesteld worden aan hoge niveaus van (niet) pathogene stoffen, die het immuunsysteem kunnen activeren en vervolgens de eiwit- en aminozuurstofwisseling beïnvloeden. Kwantitatieve informatie over de aminozuurbehoefte van varkens met een geactiveerd immuunsysteem is schaars. Deze informatie stelt voerfabrikanten in staat om varkensvoer te optimaliseren op basis van variatie in gezondheidsstatus waarmee, door het verstrekken van aangepaste voeding, de dierprestaties verbeterd kunnen worden. Voor de implementatie van doelgerichte voerstrategieën is het classificeren van bedrijven op basis van gezondheidsstatus echter cruciaal. Daarnaast zijn onderzoeksmodellen nodig waarmee het effect van immuunsysteem activatie op de aminozuurstofwisseling onderzocht kan worden, die relevant zijn voor het contrast in gezondheidsstatus dat tussen commerciële varkensbedrijven wordt waargenomen. Daarnaast zijn onderzoekstechnieken vereist waarmee veranderingen in aminozuurstofwisseling kunnen worden gekwantificeerd. Het doel van dit proefschrift was om het effect van gezondheidsstatus op de aminozuurbehoefte voor aanzet van lichaamseiwit en voor het functioneren van het immuunsysteem van vleesvarkens te kwantificeren.

In de studie beschreven in **Hoofdstuk 2** is een gezondheidsstatus web ontwikkeld als concept waarmee vleesvarkensbedrijven geclassificeerd kunnen worden, gebaseerd op data die geregistreerd wordt op commerciële bedrijven. Zes kengetallen werden in het gezondheidsstatus web opgenomen, waaronder gemiddelde groei (per dier per dag), EW-conversie, uitval, incidentie van pleuritis en percentage van afgekeurde long en lever op basis van slachtgegevens, over een periode van één jaar. Technische resultaten van 1074 en 783 Nederlandse vleesvarkensbedrijven en gegevens van de slachterij van 50208 en 47426 bedrijfsleveringen, verkregen over respectievelijk 2011 en 2012, werden gebruikt als data representatief voor de Nederlandse populatie van vleesvarkensbedrijven. Voor elk bedrijf werd een score per kengetal berekend door middel van inter- en extrapolatie waarbij de data representatief voor de Nederlandse populatie van vleesvarkensbedrijven als referentie werd gebruikt. De gemiddelde score van de zes kengetallen werd gebruikt om bedrijven te classificeren als hebbende een suboptimale, conventionele of hoge gezondheidsstatus. Het gezondheidsstatus web kan toegepast worden door voerfabrikanten in de ontwikkeling van doelgerichte voerstrategieën waarmee door het verstrekken van aangepaste voeding die aansluit bij de aminozuurbehoefte van varkens met eenzelfde gezondheidsstatus de productie-efficiëntie verbeterd kan worden.

In de studies beschreven in **Hoofdstuk 3, 4 en 5** zijn technieken ontwikkeld om de aminozuurbehoefte te bepalen en om veranderingen in aminozuurstofwisseling aan te tonen. Uit de studie beschreven in Hoofdstuk 3 bleek dat de dosisrespons techniek een geschikte techniek is voor het bestuderen van de aminozuurbehoefte met een aflopend Lysine (Lys) aanbod in de tijd, Lys titratiestappen van 3 dagen elk en N excretie in urine als respons criterium. De dosisrespons techniek is een simpele, accurate techniek waarmee veranderingen in aminozuurbehoefte van individuele maaltijd gevoerde varkens gekwantificeerd kan worden. Echter, een minimale periode van 21 dagen is vereist voor elk individuele varken (titratiestappen van 3 dagen maal ten minste 7 verschillende aminozuurniveaus). Dit maakt deze techniek ongeschikt in situaties waarin het moeilijk is een evenwicht te behouden, bijvoorbeeld bij het bestuderen van het effect van immuunsysteem activatie op de aminozuurbehoefte. Daarnaast kan met een dosisrespons studie de aminozuurbehoefte van één aminozuur per keer geschat worden, er wordt geen inzicht verkregen in de gelijktijdige veranderingen in het verbruik van andere aminozuren. De irreversibele verliezen (ILR, de hoeveelheid vrije aminozuren die verdwijnen uit de plasma pool per tijdseenheid voor eiwit synthese of voor oxidatie van aminozuren) kunnen gelijktijdig van meerdere aminozuren bepaald worden. Hierdoor kan een verschuiving in stofwisseling van verschillende aminozuren worden aangetoond. De gecombineerde metingen van de N aanzet in het lichaam, de hoeveelheid ILR van aminozuren uit het plasma, de ureum verschijningssnelheid en ^{13}C verschijningssnelheid in plasma eiwitten (na een bolus van ^{13}C gelabelde aminozuren en $^{15}N_2$ ureum) gaf inzicht in de consequenties van immuunsysteem activatie op de aminozuurstofwisseling.

In de studie beschreven in **Hoofdstuk 4** werden vleesvarkens van ongeveer 8 weken oud aangevoerd van een bedrijf gekarakteriseerd met een hoge gezondheidsstatus (n=14) of een suboptimale gezondheidsstatus (n=14), gebaseerd op de aanwezigheid van antistoffen in het bloed tegen specifieke pathogen en op hygiëne status. Aan de varkens werd een voerbehandeling verstrtekt met een adequaat of deficiënt aanbod aan de essentiële aminozuren methionine (Met) + cysteine (Cys), threonine (Thr) en tryptofaan (Trp). Bij de varkens met een suboptimale gezondheidsstatus werden hogere serum haptoglobine waarden, lagere serum albumine waarden en een hoger aantal leukocyten geconstateerd dan bij de varkens met een hoge gezondheidsstatus, duidend op een hogere mate van immuunsysteem activatie bij de start van het experiment. De schijnbare fecale droge stof en N verteerbaarheid van de rantsoenen was lager in varkens met een suboptimale gezondheidsstatus dan in varkens met een hoge gezondheidsstatus. Varkens met een suboptimale gezondheidsstatus die een adequaat aminozuuraanbod via het voer verstrekt kregen, vertoonden echter compensatoire groei na aankomst, geassocieerd met een hogere N retentie en grotere efficiëntie waarmee verteerbaar N aangezet werd in vergelijking met varkens met een hoge gezondheidsstatus. Varkens met een suboptimale gezondheidsstatus hadden een lagere plasma ILR voor Lys en een grotere ureum pool grootte dan varkens met een hoge gezondheidsstatus, indicatief voor een hogere aminozuuroxidatie door ongebalanceerde aminozuren. Dit was met name het geval in varkens met een suboptimale gezondheidsstatus die het deficiënte aminozuuraanbod via het voer

verstrekt kregen. De resultaten suggereren dat er een grotere competitie bestaat tussen het gebruik van aminozuren voor groei en lichaamseiwitaanzet enerzijds en gebruik voor het adequaat functioneren van het immuunsysteem anderzijds bij een deficiënt aminozuuraanbod van Met + Cys, Thr en Trp via het voer.

In de studie beschreven in **Hoofdstuk 5** ontvingen in totaal 16 borgen een adequaat of een beperkt eiwitaanbod via het voer en werden allen gechallenged met een intraveneuze toediening van complete Freund's adjuvant (CFA) om een systemische longontsteking te induceren. Serum acute fase eiwitten, N retentie meting en de ILR van acht aminozuren werden pre- en post-challenge bepaald. CFA leidde tot immuunsysteem activatie, blijkend uit een twee- tot viervoudige verhoging in serum concentraties aan acute fase eiwitten. De CFA challenge verhoogde de N excretie in urine en tendeerde naar een verlaagde N retentie in varkens die een adequaat eiwitaanbod verstrekt kregen, maar niet in varkens die een beperkt eiwitaanbod verstrekt kregen. De grootte van de Met pool was ongeveer 230% groter op dag 8 post-challenge dan op dag 3 post-challenge in varkens die een adequaat eiwitaanbod verstrekt kregen. De ILR voor valine (Val) was lager op dag 8 dan op dag 3 post-challenge. De waargenomen veranderingen in aminozuurstofwisseling indiceren dat met name Trp limiterend kan worden bij immuunsysteem activatie, terwijl een Lys in overmaat aanwezig komt (**Algemene Discussie**). Bovendien lijkt het verbruik van Met, tyrosine (Tyr) en Val voor het functioneren van het immuunsysteem verhoogd in varkens met een systemische longontsteking.

Het aanbod aan aminozuren of eiwit via het voer kan de acute fase response beïnvloeden, hieruit blijkt de noodzaak van het verstrekken van een adequaat aminozuuraanbod via het voer voor een passend functioneren van het immuunsysteem van vleesvarkens (Hoofdstuk 4 en 5).

Voordat implementatie van doelgerichte voerstrategieën voor bedrijven met eenzelfde gezondheidsstatus kan plaatsvinden is vervolg onderzoek wenselijk waarin de mogelijk gunstige effecten van het verhogen van met name Trp, Met , Tyr en Val ten opzichte van Lys voor het functioneren van het immuunsysteem en voor lichaamseiwitaanzet in varkens van bedrijven met een suboptimale gezondheidsstatus worden onderzocht.

Acknowledgements

Na een intensieve maar vooral leerzame periode van meer dan vier jaar is het zo ver. De laatste woorden van dit boekwerk komen op papier en die besteed ik graag aan het bedanken van alle mensen die mij met dit levenswerk geholpen hebben.

Ik begin bij het begin, Frankie bedankt dat je me hebt overtuigd om te beginnen aan mijn PhD. Je hebt me de kracht gegeven om door te zetten, ik denk nog vaak aan je.

Een PhD doe je niet alleen, ik heb samen mogen werken met een team van medestrijders waarvan ik ontzettend veel geleerd heb. Alfons, jij hebt me geleerd om niet altijd alle ballen in de lucht te houden, maar ook af en toe wat te laten landen. Je wist me met je kritische blik op het juiste pad te houden wanneer ik afdwaalde. Walter, bedankt voor je prikkelende manier van supervisie. Je hebt me aangestoken met het maken van (ingewikkelde) spreadsheets (wat tussen haakjes staat kun je net zo goed weglaten ☺). Je "Ja, en" mentaliteit heeft me geleerd verder te kijken en om uiteindelijk "Ja, of" te zeggen, beide hebben zeker bijgedragen aan de kwantiteit en de kwaliteit van het boekje. Joost, bedankt voor al je input, gelabeld en ongelabeld, ik heb er heel veel aan gehad! Carola, naast je kennis op wetenschappelijk gebied was jou praktijkgerichte ervaring in de varkenshouderij zeer nuttig, bedankt daarvoor. Hetty, ik ken niet veel mensen die zo snel kunnen praten en ook echt iets zinnigs zeggen. Met je netwerk was je de lijm van het spinnenweb. P.s. het peper en zout stel werkt ook nog steeds! Het aantal supervisors was wat aan de hoge kant, toch heb ik van ieder van jullie heel veel kunnen leren en kon ik bij jullie terecht als het nodig was. Wouter Hendriks bedankt voor het verstrekken van een goede werksfeer en voor je oog voor detail, ik doe graag nog een keer een weddenschap met je...

Paranymph Panos, you were the help in times of need. Thank you for all your hard work, for sharing your knowledge on nutrition and immunology, and for all the fun we had! I really enjoyed working with you. When you put two chaotic persons together they will become more organized! You taught me how to keep overview, and when I need to glue fecal bags again I know where to find you! Yvonne bedankt voor je betrokkenheid in het project en succes met het afronden van je eigen PhD.

Nathalie, thank you for your hospitable welcome at INRA, and for giving me the opportunity to learn from your work. I really enjoyed my short trip to France.

Michel bedankt voor het harde werk op het lab. Jij hebt vele malen goed gemaakt wat de Speedvac liet afweten. Dit geldt ook voor Erika, Jane-Martine, Leon, Rick, Saskia, en Xuan. Betty en Yvonne bedankt voor het regelen van het papierwerk rondom het promoveren. Het heeft me veel ritjes naar Wageningen bespaard.

Ik wil het Productschap Diervoeder en het Productschap Vee, Vlees en Eieren bedanken voor de medefinanciering van dit project. Alle leden van de stuurgroep Bedrijfsspecifieke Voeding wil ik bedanken voor het afreizen naar onze vele bijeenkomsten en jullie praktijkgerichte bijdrage. Ook de varkenshouders die betrokken waren in het project wil ik bedanken voor hun bijdrage, en de dierenartsen

voor het invullen van een enquête voor het definiëren van de gezondheidsstatus van vleesvarkensbedrijven. Ik wil betrokkenen vanuit de mengvoerindustrie en van Vion Food Group bedanken voor het verstrekken van data voor Hoofdstuk 1, soms moest ik wat geduld hebben, maar dan heb je ook wat.

De mensen van DB (Centraal Veterinair Instituut, Lelystad) waaronder Albert, Andre, Gerald, Gerard, Gerrit Jan, Henk en Jan wil ik bedanken voor hun hulp bij de uitvoering van de proeven in Hoofdstuk 3 en 5. De watergevechten vergeet ik nooit meer! Gerrit Jan, bedankt voor je begeleiding en nuchtere kijk tijdens mijn eerste echte dierproef. Ruud en Piet bedankt voor jullie ondersteuning bij de dierproeven en Piet voor zijn pipetteer techniek! Ik wil Dirk en Jan bedanken voor het opereren van de varkens onder soms suboptimale omstandigheden en voor hun veterinaire kennis. Hans van Diepen, ik heb veel geleerd door samen met je de proefvoeders samen te stellen, iets dat elke keer weer een uitdaging is. Aura bedankt voor de leuke gesprekken in ons gezamenlijke kantoor en voor het wateren van de plantjes in mijn afwezigheid. Alle mensen van de proefaccommodatie de Haar (Wageningen), in het bijzonder Ben, Bjorge, Jarno, Ries en Sander bedankt voor het harde werk en voor jullie praktische inzicht, voor het ombouwen van de stallen met hygiëne sluizen en het inrichten van de operatieruimte voor de dierproef die in twee verschillende stallen plaats vond (Hoofdstuk 4) en de pilotstudies. Ook Ilona, Marcel, Sven, en Tamme bedankt voor alle voorbereidingen en het aanpoten in de respiratiecellen inclusief zweetpakken, handschoenen en mondmaskers. Dayenne, Janneke, Matthew, Sabrina, Suzan, Sander en Yvonne bedankt voor jullie getoonde interesse en enthousiasme in dit onderwerp en de bijdrage aan dit proefschrift, voor de hulp bij de dierproeven in de varkensstallen en voor de gezellige momenten samen. Ik vond het leuk en leerzaam om met jullie samen te werken.

Sharon, bedankt voor het doorspitten van mijn referentielijst en voor alle hulp, zelfs tot in de late uurtjes! Han ook bedankt voor de finale check en voor het doorspitten van al deze vaktaal..... T en T bedankt voor de oppas, gezelligheid en Tessa bedankt voor het ontwerpen van de varkentjes op de voorkant van het boekje!

Anke van Brakel, bedankt dat ik zelfs na mijn studie weer bij je kon aankloppen voor onderdak! Het heeft me heel veel reisuurtjes gescheeld.

Dit boekwerk is zeker mede mogelijk gemaakt door Roy. Zonder je onvoorwaardelijke liefde was dit proefschrift er niet geweest. Liefde is het afstaan van je soeplepel, die echt heilig is voor een Kampman, voor het opvangen van varkensurine. Bedankt voor al je steun en je geduld, zelfs op momenten dat het vast heel moeilijk voor je was, bijvoorbeeld telkens als ik de BMW weer in een zwijnenstal had omgetoverd. Ik ga er aan werken ;) Ook wil ik mijn schoonouders bedanken, Bertus je boerenwijsheid komt nog steeds van pas. Ik wil mijn ouders bedanken voor het geven van onvoorwaardelijke liefde en voor alle han en span diensten in tijden van drukte. Familie en vrienden bedankt voor jullie luisterende oren en de gezelligheid! Femke, Lara, Bibi, Eef en Femke Z, bedankt voor de nodige afleiding! Tot slot wil ik mijn dochter Lynn bedanken voor het weerspiegelen van karaktereigenschappen en voor haar kunst om van de kleinste dingen te genieten, het werkt aanstekelijk.

About the author

Curriculum Vitae

Esther Kampman – van de Hoek was born on 20 August 1982 and grew up in Ommen, The Netherlands. She finished secondary school at the Vechtdal College (Hardenberg, The Netherlands) in 1999, whereafter she started her study Paraveterinary at the Groenhorst College (Barneveld, The Netherlands). In 2001 she started her study Animal Management at the Van Hall Instituut (Leeuwarden, The Netherlands), and obtained her Bachelor degree in 2004. From 2005 up to 2006 she worked fulltime as a nutritionist at Zodiac Zoos (Epe, The Netherlands). In 2006 she started her study Animal Sciences at Wageningen University and specialized in Animal Nutrition and Adaptation Physiology. For the specialization in Animal Nutrition she examined the effect of dietary fibre in dog feed on feed intake and behaviour, in collaboration with Gent University, Belgium. For the specialization in Adaptation Physiology she investigated the effect of fish oil supplementation to sows during gestation and lactation on the growth, learning ability, and behaviour of piglets. She participated in a summer course Organic Farming at Hohenheim University (Stuttgart, Germany). After her graduation in 2008 she assisted in pig nutritional and behavioural studies at the Adaptation Physiology Group at Wageningen University. In 2009, she started her PhD at Wageningen UR Livestock Research and Wageningen University, commissioned by the Product Board Animal Feed (PDV) and the Product Board for Livestock, Meat and Eggs (PVE). The PhD thesis was aimed at quantifying the nutrient requirements of pigs as affected by health status. The results of the PhD are presented in the present thesis. In 2013 she was awarded the NZV Travel Grant for the best paper presentation by the Nederlandse Zootechnische Vereniging (NZV). Since 2014 Esther is employed as a researcher pigs at the Agrifirm Innovation Center of Agrifirm (Apeldoorn, The Netherlands).

List of publications

Peer reviewed scientific publications

Bosch G, Beerda B, van de Hoek E, Hesta M, van der Poel AFB, Janssens GPJ, Hendriks WH (2009) Effect of dietary fibre type on physical activity and behaviour in kennelled dogs. *Appl Anim Behav Sci* **121**, 32-41.

Kampman - van de Hoek E, Gerrits WJJ, van der Peet-Schwering CMC, Jansman AJM, van den Borne JJGC (2013) A simple amino acid dose-response method to quantify amino acid requirements of individual meal-fed pigs. *J Anim Sci* **91**, 4788-4796.

Kampman - van de Hoek E, Sakkas P, Gerrits WJJ, van den Borne JJGC, van der Peet-Schwering CMC, Jansman AJM (2015) Induced lung inflammation and dietary protein supply affect N retention and amino acid metabolism in growing pigs. *Br J Nutr* **113**, 414-425.

Kampman - van de Hoek E, Jansman AJM, van den Borne JJGC, van der Peet-Schwering CMC, van Beers-Schreurs H, Gerrits WJJ. Dietary amino acid deficiency reduces the utilization of amino acids for growth in growing pigs following a period of low health as characterised by antibody presence and hygienic environment. Submitted to *Journal of Nutrition*.

Kampman - van de Hoek E, Sakkas P, Gerrits WJJ, van Beers-Schreurs H, van der Peet-Schwering CMC, van den Borne JJGC, Jansman AJM. A novel scoring system for the classification of the health status of growing-finishing pig farms. To be submitted.

Conference proceedings and abstracts

van de Hoek E, Borgijink S, van den Borne JJGC, Gerrits WJJ, Jansman AJM, van der Peet-Schwering CMC (2010) Titration studies to determine amino acid requirements of individual growing pigs. In *Proceedings of the 3th international symposium on energy and protein metabolism and nutrition*, 6-10 Sep 2010, Parma, Italy, **127**, 115-116.

van de Hoek E, Gerrits WJJ, van der Peet-Schwering CMC, Jansman AJM, van den Borne JJGC (2011) Development of a dose-response technique to determine amino acid requirements of individual growing pigs. In *36th Animal Nutrition Research forum*, 19 April, Heverlee, Belgium. pp. 51 - 52.

van de Hoek E, van den Borne JJGC, Gerrits WJJ, van der Peet-Schwering CMC, Jansman AJM (2011) Health status and amino acid requirements in pigs. In *Proceedings of*

the international symposium "Nutrition and sustainable pig production", 9 June 2011, Wageningen, The Netherlands.

van de Hoek E, van den Borne JJGC, Gerrits WJJ, van der Peet-Schwering CMC, Jansman AJM (2011) Evaluation of two models for immune system stimulation in pigs. In *Proceedings of the Oskar Kellner symposium on metabolic flexibility in animal and human nutrition*, 9-11 September 2011, Warnemünde, Germany.

Kampman - van de Hoek E, Gerrits WJJ, van den Borne JJGC, van der Peet-Schwering CMC, van Beers-Schreurs H, Jansman AJM (2013) Challenge models to study the effect of immune system activation on amino acid metabolism in pigs. In *Proceedings of the 4th international symposium on energy and protein metabolism and nutrition*, 9-12 Sep 2013, Sacramento, USA, **134**, 237-238.

Kampman - van de Hoek E, Sakkas P, van den Borne JJGC, Gerrits WJJ, van der Peet-Schwering CMC, van Beers-Schreurs H, Jansman AJM (2013) Impact of CFA and dietary protein supply on acute phase responses and nitrogen retention in pigs. In *Proceedings of the 4th international symposium on energy and protein metabolism and nutrition*, 9-12 Sep 2013, Sacramento, USA, **134**, 367-368.

Other Publications

van de Hoek E, Gerrits WJJ, van den Borne JJGC, van der Peet-Schwering CMC, Jansman AJM (2011) Effect van energieaanbod op de optimal lysine : energie verhouding in voer van vleesvarkens. Validatie van de binnen-diertitratietechniek. Vertrouwelijk rapport 269, Wageningen UR Livestock Research, The Netherlands.

van de Hoek E, Gerrits WJJ, van den Borne JJGC, van der Peet-Schwering CMC, Jansman AJM (2011) Ontwikkeling van de isotoopverdunningstechniek bij varkens: plasmametingen met ^{13}C gelabelde aminozuren en ^{15}N ureum. Vertrouwelijk rapport 270, Wageningen UR Livestock Research, The Netherlands.

Kampman – van de Hoek E, Gerrits WJJ, van den Borne JJGC, van der Peet-Schwering CMC, van Beers-Schreurs H, Jansman AJM (2012) Selectie van een onderzoeksmodel voor het bestuderen van de invloed van immuunsysteem activatie op de aminozuurbehoefte van varkens. Vertrouwelijk rapport 335, Wageningen UR Livestock Research, The Netherlands.

Training and Supervision Plan[1]

Description	Year
The Basic Package (3 ECTS[2])	
WIAS Introduction Course	2009
Course on philosophy of science and/or ethics	2009
International conferences (4 ECTS)	
3rd Int. Symp. on Energy and Protein Metabolism and Nutrition, Parma, Italy	2010
Oskar Kellner Symposium on Metabolic Flexibility in Human and Animal Nutrition, Warnemünde, Germany	2011
Nutrition and sustainable pig production, Wageningen, The Netherlands	2011
Developments in Phosphorus Nutrition in Pigs and Poultry, Wageningen, The Netherlands	2012
4th Int. Symp. on Energy and Protein Metabolism and Nutrition, Sacramento, USA	2013
Seminars and workshops (3 ECTS)	
WIAS Science Day, Wageningen, The Netherlands	2009
Nutritionele en infectieuze factoren die de groei beinvloeden, Ewijk, The Netherlands	2009
Int. Symposium Poultry Nutrition to manage future challenges, Wageningen, The Netherlands	2009
Int. Symposium Progress in pig nutrition: health, envorinment and metabolism, Lelystad, The Netherlands	2009
WIAS Science Day, Wageningen, The Netherlands	2010
35th ANR Forum, Lelystad, The Netherlands	2010
Dietary lysine and the importance of processing food- and feedstuffs seminar, Wageningen, The Netherlands	2010
Mini symposium: How to write a world-class paper, Wageningen, The Netherlands	2010
Scientific Research in Animal Welfare: Do We Make a Difference? Wageningen, The Netherlands	2011
WIAS Science Day, Wageningen, The Netherlands	2011
36th ANR Forum, Leuven Belgium	2011
PDV Themabijeenkomst "Voeding en Darmgezondheid", Wageningen, The Netherlands	2011
Seminar Learning how to eat like a pig, Wageningen, The Netherlands	2011
WIAS Science Day, Wageningen, The Netherlands	2013

[1]Completed in fulfilment of the requirements for the education certificate of the Graduate School WIAS (Wageningen Institute of Animal Science).
[2]One ECTS (European Credit Transfer System) equals a study load of 28 hours.

Presentations (6 ECTS)

Oral, Int. Symp. on Energy and Protein Metabolism and Nutrition, Parma, Italy	2010
Poster, WIAS Science Day, Wageningen, The Netherlands	2011
Oral, ANR Forum, Leuven, Belgium	2011
Oral, Nutrition and sustainable pig production, Wageningen, The Netherlands	2011
Oral, Oskar Kellner Symposium on Metabolic Flexibility in Human and Animal Nutrition, Warnemünde, Germany	2011
Poster, 4th European Symposium on Porcine Health Management, Bruges, Belgium	2012
Oral, WIAS Science Day, Wageningen, The Netherlands	2013
Poster, 4th Int. EAAP Symp. on Energy and Protein Metabolism and Nutrition, Sacramento, USA	2013

In-Depth Studies (7 ECTS)

Advanced Immunology Course, Utrecht University, The Netherlands	2011
Orientation on mathematical modelling in biology, Wageningen, The Netherlands	2011
WIAS Design of Experiments, Wageningen, The Netherlands	2009
Statistics for the Life Sciences, Wageningen, The Netherlands	2010
Analytical work and possibilities within animal nutrition sciences, Wageningen, The Netherlands	2009

Professional Skills Support Courses (4 ECTS)

Techniques for Scientific Writing, Wageningen, The Netherlands	2010
Teaching and supervising Thesis students, Wageningen, The Netherlands	2011
Project and Time Management, Wageningen, The Netherlands	2010

Research Skills Training (6 ECTS)

Preparing own PhD research proposal	2009

Didactic Skills Training (9 ECTS)

Lecturing Veldwerkprakticum Gymnasiumleerlingen Pantarijn, Sprint-up project, Wageningen, The Netherlands	2010-2011
Supervising 4 MSc students	2009-2012

Organisation of seminars and courses (1 ECTS)

Organizing WIAS Science Day 2010, Wageningen, The Netherlands	2009-2010

Education and Training Total	**43 ECTS**

List of abbreviations

AA	amino acids
ACTH	adrenocorticotropic hormone
ADG	Average daily gain
AGP	α1-acid glycoprotein
ApoA1	apolipoprotein A1
APP	acute phase proteins
B	AA release from body protein breakdown
BCAA	branched-chained AA
BRA	*Brachyspira dysentery*
BW	body weight
CFA	Complete Freund's Adjuvant
CRP	C-reactive protein
ECF	ethyl chloroformate ester
ECR	energy conversion ratio
ECTS	European Credit Transfer System
EMCV	encephalomyocarditis virus
EW	energy intake (Dutch: energie waarde)
GSH	glutathione present in cells as thiol-reduced glutathione
GSSG	disulfide-oxidized glutathione
HAPI	health status acute phase index
HHS	high health status
HSF III	hepatocyte stimulating factor
I	AA absorption from the diet
i.m.	intramuscular
i.v.	intravenous (*i.v.*)
IAAO	indicator AA oxidation
IDO	indoleamine 2,3 dioxygenase
IL	interleukin
ILR	irreversible loss rate
LHS	low health status
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
MSPE	mean square prediction error
MYC	*Mycoplasma hypneumoniae*
N	nitrogen
NAPI	nutritional acute phase index
O	AA oxidation
PCV2	Porcine Circovirus type 2
Pig-MAP	Pig major acute-phase protein
PRRSV	Porcine reproductive and respiratory syndrome virus
Q	the turnover of AA in plasma
S	AA use for protein synthesis
s.c.	subcutaneous
SAA	serum amyloid A
SD	standard deviation
SE	standard error
SPF	specific pathogen free
STREP	*Streptococcus suis*
TDO	Trp 2,3-dioxygenase
TNF	tumour necrosis factor
TO	turpentine oil
TTR	tracer-to-tracee ratio
WBC	white blood cells
WIAS	Graduate School of Wageningen Institute of Animal Sciences

Colophon

The research described in this thesis was financially supported by the Product Board Animal Feed, the Product Boards for Livestock and Meat, Wageningen University and Wageningen UR Livestock Research.

Financial support from Agrifirm Innovation Center (Apeldoorn) for printing this thesis is gratefully acknowledged.

Cover design T. van de Hoek – Paarhuis and E. Kampman – van de Hoek
Thesis design E. Kampman – van de Hoek
Printed by GVO drukkers & vormgevers B.V. | Ponsen & Looijen, Ede, The Netherlands