Antibodies and longevity of dairy cattle: genetic analysis

Britt de Klerk

Thesis committee

Promotor
Prof. Dr J.A.M van Arendonk
Professor of Animal Breeding and Genetics
Wageningen University

Co-promotors
Dr J.J. van der Poel
Assistant professor, Animal Breeding and Genomics Group
Wageningen University

Dr Ir B.J. Ducro
Assistant professor, Animal Breeding and Genomics Group
Wageningen University

Other members
Prof. Dr H.F.J. Savelkoul, Wageningen University, Wageningen, The Netherlands
Prof. Dr F.S. Schenkel, University of Guelph, Guelph, Canada
Dr B. Heringstad, Norwegian University of Life Sciences, Aas, Norway
Prof. Dr Y. Schukken, De Gezondheidsdienst voor Dieren, Deventer, The Netherlands

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

Antibodies and longevity of dairy cattle:

Genetic analysis

Britt de Klerk

Thesis

submitted in fulfillment of the requirements for the degree of doctor at
Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday June 10, 2016
at 4 p.m. in the Aula

De Klerk, B.
Antibodies and longevity of dairy cattle: genetic analysis.
136 pages

PhD thesis, Wageningen University, the Netherlands (2016)
With references, with summaries in English and Dutch

ISBN 978-94-6257-758-9

Abstract

De Klerk, B. (2016). Antibodies and longevity of dairy cattle: genetic analysis.
PhD thesis, Wageningen University, the Netherlands

In dairy cattle longevity is an important trait. Longevity is strongly related to disease resistance, since a more healthy cow is expected to realize a longer productive life (longevity). Information on longevity records, however, can only be measured after an animal has been culled. Therefore, predictors of longevity traits are needed in order to breed for improved longevity. This thesis aimed to gain more knowledge and genetic background on (natural) antibodies as predictors for longevity. Both natural antibody- and specific antibody levels were studied. Antibody binding the naive antigen KLH was assumed to be natural antibody. Antibodies binding bacteria-derived antigens LTA, LPS and PGN were assumed to be specific antibodies. It was shown that natural antibody levels measured in milk and blood are genetically high correlated (± 0.80) for the two studied isotypes (IgG and IgM). On the other hand, phenotypically, natural antibodies (from both IgG and IgM isotype) measured in milk cannot be interpreted as the same trait (phenotypic correlation = ± 0.40). Antibodies (both natural-and specific antibodies) showed a negative relation with longevity: first lactation cows with low IgM or IgG levels were found to have a longer productive life. When using estimated breeding values for longevity, only a significant relation was found between natural antibody level (IgM binding KLH) and longevity. Lastly, this thesis reports on a genome-wide-association study (GWAS), to detect genes contributing to genetic variation in natural antibody level. For natural antibody isotype IgG, genomic regions with a significant association were found on chromosome 21 (BTA). These regions included genes that play an important role in isotype class switching (from IgM to IgG). The gained knowledge on relations between antibodies and longevity and the gained insight on genes responsible for natural antibodies level make antibodies potential predictors for longevity.

Contents

1

General Introduction

1.1 Importance dairy industry

The dairy industry contributes substantially to the world economy. Nowadays, about 150 million households around the world are involved in milk production (FAO, 2016). Milk and its products are an important source of food. To fulfil the demand of food with the increase of human population in the future, the dairy sector needs to respond in a sustainable manner. In the Netherlands, the dairy industry is the largest sector within the Dutch agriculture. It accounts for about 20% of all agricultural incomes. Currently, around 15.000 farms in the Netherlands are specialized dairy farms, housing about 1.5 million dairy cows (CRV, jaarstatistieken, 2015). The number of cows reduced over time while the individual milk production of cows increased as shown in Figure 1.1. Breeding has made a substantial contribution to the higher milk production as well as improvements in feeding and management. Breeding programs at the end of the last century mainly focused on production and fertility traits (Nebel and Jobst 1998), while little attention was given to health traits. During the last decades more attention has been paid to health traits in breeding programs in order to breed for a balanced cow which not only has a good production but also good fertility traits, and good health status and thus comprising all prerequisites for a long productive life.
Another trend seen during last decades is that the number of farms has decreased while the number of cows per farm increased. As a consequence, less attention can be paid to each individual cow by the farmer. This might compromise good dairy farming practice which is aiming at on-farm production of safe, and high-quality milk from healthy animals under acceptable conditions (FAO, 2016).

1.2 Breeding programs

In order to obtain genetic gain and improve desirable traits, there is a constant need for optimization of breeding programs and search for new useful parameters to be included within breeding programs. Breeding programs are used to realise genetic improvement in desired traits. The desired direction of change is summarized in the breeding goal. Selection of bulls and cows to realise an improvement in the breeding goal is based on estimated breeding values. Estimated breeding values are based on phenotype records on the animal itself and on relatives. In estimating breeding values, the phenotypic information is combined with pedigree information and information on genetic parameters (heritabilities and correlations).

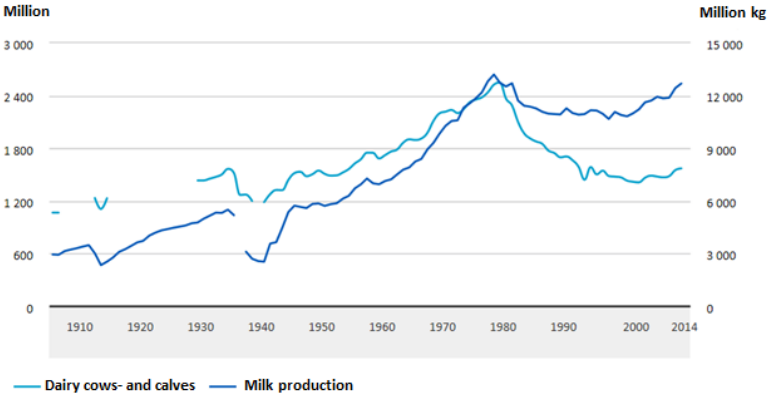

Figure 1.1: Number of dairy cows- and calves and their total milk production in million kilograms over the past 100 years.

The selection response in individual traits, however, is relatively low due to the complexity of most traits. Genetically, traits can be classified as either monogenic or complex traits. Monogenic traits are strongly predisposed by variation within a single gene. Complex (or quantitative) traits are thought to result from variation within multiple genes and their interaction with behavioural and environmental factors. Many different genes are affecting complex traits, with one gene capturing only a limited proportion of the total genetic variation (Hayes and Goddard, 2001; Van Raden et al. 2009). For most complex traits, such as production and health traits, the environment accounts for more variation in the animal's performance than genetics (Goddard and Hayes, 2009). Consequently, relatively small gains were achieved by breeding schemes based on phenotypes. The rate of genetic gain can be improved by genomic selection (Hayes et al., 2009). In genomic selection information is used to trace the inheritance of chromosomal segments. Furthermore, in genomic selection knowledge on contribution of individual genes to genetic variation can be exploited.

1.3 Longevity

In dairy longevity is of great importance, both from an economical and an animal welfare point of view (Allaire and Gibson, 1992; Thomsen and Houe, 2006; Pritchard et al. 2013). Generally, longevity refers to the length of life that ends with a natural death. Production animals, however, are mostly slaughtered before they have the opportunity to die from a natural cause. Currently, in the Netherlands, the average age in years of dairy cows at slaughter is approximately 5.7 (CRV,

Jaarstatistieken, Arnhem, The Netherlands, 2015), equivalent to about 3.5 lactations. Longevity in dairy cows is mostly defined as productive life. Productive life can be defined as the time in days between first calving and last day in production. For dairy cows there is a need to further improve the average productive lifetime. Cows with a longer productive life, contribute to a more efficient and sustainable production (Strandberg and Sölkner, 1996). The main reason for a short productive lifetime is involuntary culling due to poor health or fertility issues. From not only an economic but also an animal welfare point of view, it is beneficial that a cow can maintain a longer, healthy productive life.
Productive life is a complex trait which is influenced by many different aspects like: fertility, calving ease, conformation, somatic cell count, lactation persistency, and lameness (Pfeiffer et al. 2015). Low heritability of longevity traits, including productive lifetime, suggests that genetic improvement of this trait is a slow process (Van Pelt et al. 2015). Therefore, to gain higher genetic improvement it is important to develop alternative predictors for longevity.

1.4 Health in dairy cows

As mentioned before, the last decades dairy farming has changed enormously. In the last decade the number of cows per farm increased with 40% in The Netherlands (CRV, Jaarstatistieken, Arnhem, The Netherlands 2014), making dairy farming more intensive. The ideal dairy cow, therefore, is a cow that is productive during a long productive life , which implies that the cow has no fertility issues, and does not suffer from any diseases. Diseases that affect dairy cattle are associated with increased risk of involuntary culling and substantial economic losses (Huijps et al., 2008; Hogeveen et al., 2011, Heikkila et al., 2012). Major health problems that occur in dairy cattle are mastitis, fertility and lameness (Huxely, 2013). Factors that contribute to economic loss due to health problems, include decreased milk production, veterinary treatment, discarded milk, increased risk of culling and increased disease risk in the future (Bradley, 2002; Zwald et al., 2006). Selection for milk production only is negatively associated with dairy health and reproductive performance (Rauw et al., 1998; Heringstad et al., 2005b; Shook, 2006; Berry et al., 2011). Dairy cattle breeding, therefore, should not only focus on production but also on health and longevity. Health is a complex trait and is influenced by different factors. Longevity is highly influenced by health traits. Biomarkers are used to measure diseases- or health-status related traits. Somatic cell count (SCC) is a regularly used biomarker related to mastitis (Samoré et al., 2003). This biomarker is

used in many dairy cattle improvement programs. Biomarkers for other health traits are needed to give a better prediction of health status.

1.5 Antibodies

Antibodies are an important part of the immune system. Antibodies can be divided into 2 major groups; natural antibodies (NAb) and specific antibodies (SpAb). Natural antibodies have a unique role in the immune system; they are polyreactive antibodies and are responsible for a first line of defence against invading pathogens that have not been encountered previously (Ochsenbein and Zinkernagel, 2000). Natural antibodies are produced by B1 B-cells (Baumgarth, 2013). B-1 cells are positively selected to recognize self-antigens, making NAb generally directed to both auto-antigens and common microbial structures like Pattern-Associated Molecular Patterns (PAMP) (Avrameas, 1991). Specific antibodies, on the other hand, are produced by B2 B-cells in response to antigen exposure. As a consequence SpAb are more restricted in their epitope recognition compared to NAb. Both natural- and specific antibodies can be involved by initial recognition and opsonisation of pathogens, resulting in complement dependent killing or phagocytosis (Hangartner et al., 2006; Ehrenstein and Notley, 2010).
Associations between antibodies and diseases have been reported in different species. In dairy cows, natural- and specific antibodies, measured in both milk- and blood samples, are associated with the prevalence and risk of mastitis (Ploegaert et al. 2008, Thompson-Crispi et al., 2013). Moreover, in chicken, high natural antibody levels, measured in blood, were associated with a longer survival period in laying hens (Sun et al., 2011). Based on these findings, natural- and specific antibodies are potential indicators for the health-status, and thus longevity, of dairy cows.

1.6 “Weerbaarvee”

The research performed in this thesis is partly based on the Dutch project ‘Weerbaar Vee’ (Resilient Livestock), which was funded by The Dutch Ministry Economic Affairs (The Hague, The Netherlands), Productschap Zuivel (Dutch Dairy Product Board, Zoetermeer, the Netherlands), CRV (Arnhem, The Netherlands), LTO-Noord Fondsen (Zwolle, The Netherlands), Animal Health Services (Deventer, The Netherlands) and Wageningen University (Wageningen, The Netherlands). The core idea of this project was to make natural resistance in dairy cows measurable, enabling improvement of overall resistance in dairy cows. Main goal of the total

project was to strive for dairy cows that suffer as little as possible from different diseases, have no claw and fertility problems, live a long and productive live in a responsibly way. Furthermore it would be ideal if a sick or diseased cow can be detected in an early stage. The importance of healthy livestock is clear: it is essential for the cow itself, the farmer, the dairy sector and the entire society. Animals can stay more healthy by keeping them away from health hazards or to make them as resistant as possible when they do encounter infections. Within the 'WeerbaarVee' project, research was aimed at finding a balance between cow and environment. Besides the adaptive part of the immune system, which development is depending on challenges from the environment, the innate immunity is an important component. Natural antibodies are one of the most important components of innate immunity, and were therefore subject of study within the 'WeerbaarVee' project.

1.7 This thesis

An important aspect of sustainable dairy production is a good health status of cows. A good health status of dairy cows provides optimal circumstances for both cow and farmer to optimize milk production and animal welfare and to prevent economical losses. A strong and well-functioning immune system is crucial for the health status of a cow. To breed for healthier cows, however, is still a challenge which concerns many different aspects. The identification of biomarkers and the detection of genes controlling health and longevity, would not only greatly enhance the understanding of such traits but also offer the opportunity to improve breeding schemes. The objectives of this thesis therefore were :

1) To find an easy measurable disease resistance related biomarker in dairy cows (antibodies);
2) Identify the relation between antibodies and longevity;
3) Identify genomic regions that are involved in antibody production/expression.

The outline of this thesis is as follows:

Chapter 2 focusses on the comparison of natural antibodies measured in blood and measured in milk. Measuring antibodies in milk is non-invasive and less labour intensive than collecting antibodies from blood samples. Additionally, when proven to be useful, antibodies measured in milk, can be included in the routine evaluation of milk, i.e. that can be included as part of the regular milk testing system (MPR). **Chapter 3**, focusses on the phenotypic relation between antibodies (both natural- and specific antibodies) measured in milk, and productive lifetime of cows. **Chapter**

4 focusses on the relation between antibodies and estimated breeding values of productive lifetime. For all animals breeding values were estimated (EBV's) for productive lifetime, and compared to both natural- and specific antibody levels. The relation between the genetic predictor of productive life and antibody levels was studied to determine whether Ab levels are related with the expected potential of a cow's productive lifetime. **Chapter 5** focusses on identifying regions within the genome that are responsible for the production/transcription of natural antibodies. The general discussion puts the results of all chapters in a broader perspective and the main focusses are: 1) the definition of antibodies, 2) the interpretation of the relation between antibodies and longevity, and 3) possibilities of genomic selection on antibodies and the opportunity of dairy breeding based on antibody levels.

1.7 References

Allaire, F. R., and J. P. Gibson. 1992. Genetic value of herd life adjusted for milk production. J. Dairy Sci. 75:1349‑1356.

Avrameas, S. 1991. Natural autoantibodies: from ' horror autotoxicus' to ' gnothu seauton'. Immunol. Today 12: 154-159.

Baumgarth, N., 2013. Innate-like B cells and their rules of engagement. Advances in Experimental Medicine and Biology 785: 57-66.

Berry, D. P., M. L. Bermingham, M. Good, and S. J. More. 2011. Genetics of animal health and disease in cattle. Ir. Vet. J. 64(1):5.

Bradley, A. 2002. Bovine mastitis: an evolving disease. Vet. J. 164(2):116-128.

Ehrenstein, M.R., and C.A. Notely. 2010. The importance of natural IgM; scavenger, protector and regulator. Nature Reviews. 10: 778-786.

Goddard, M.E. and B.J. Hayes. 2009. Mapping genes for complex traits in domestic animals and their use in breeding programs. Nature Rev. Genet. 10: 381-391.

Hangartner, L., Zinkernagel, R.M., Hengartner, H., 2006. Antiviral antibody responses: the two extremes of a wide spectrum. Nature Rev. Immunol. 6: 231-243.

Hayes, B.J., and M.E. Goddard. 2001. The distribution of the effects of genes affecting quantitative traits in livestock. Genetic. Sel. Evol. 33:209-229.

Hayes, B.J., P.J. Bowman, A.J. Chamberlain and M.E. Goddard. 2009. Invited review: genomic selection in dairy cattle: progress and challenges. J. of Dairy Sci. 92: 433-443.

Heikkila, A. M., J. I. Nousiainen, and S. Pyorala. 2012. Costs of clinical mastitis with special reference to premature culling. J. Dairy Sci. 95(1):139-150.

Heringstad, B., Y. M. Chang, D. Gianola, and G. Klemetsdal. 2005b. Genetic association between susceptibility to clinical mastitis and protein yield in norwegian dairy cattle. J. Dairy Sci. 88(4):1509-1514.

Hogeveen, H., K. Huijps, and T. Lam. 2011. Economic aspects of mastitis: New developments. New Zealand Veterinary Journal 59(1):16-23.

Huijps, K., T. J. Lam, and H. Hogeveen. 2008. Costs of mastitis: facts and perception. J. Dairy Res. 75(1):113-120.

Huxley, J. N. 2013. Impact of lameness and claw lesions in cows on health and production. Livestock Science 156(1–3):64-70.

Ingvartsen, K. L. and K. Moyes. 2013. Nutrition, immune function and health of dairy cattle. Animal 7(Supplements1):112-122.

LeBlanc, S. J., K. D. Lissemore, D. F. Kelton, T. F. Duffield, and K. E. Leslie. 2006. Major Advances in Disease Prevention in Dairy Cattle. J. Dairy Sci. 89(4):1267-1279.

Nebel, R.L. and S.M. Jobst. 1998. Evaluation of systematic breeding programs for lactating dairy cows: a review. J. Dairy Sci. 81: 1169-1174.

Ochsenbein, A.F., Zinkernagel, R.M. 2000. Natural antibodies and complement link innate and acquired immunity. Science, 21(12):624.

Pfeiffer, C., C. Fuerst, V. Ducrocq and B. Fuerst-Waltl. 2015. Short communication: Genetic relationships between functional longevity and direct health traits in Austrian Flechvieh cattle. J. Dairy Sci. 98:7380-7383.

Pritchard, T., M. Coffey, R. Mrode, and E. Wall. 2013. Understanding the genetics of survival in dairy cows. J. Dairy Sci. 96:3296‑3309.

Rauw, W. M., E. Kanis, E. N. Noordhuizen-Stassen, and F. J. Grommers. 1998. Undesirable side effects of selection for high production efficiency in farm animals: a review. Liv. Prod. Sci. 56:15-33.

Samoré, A. B., M. D. P. Schneider, F. Canavesi, A. Bagnate, and F. A. Groen. 2003. Relationship between somatic cell count and functional longevity assessed using survival analysis in Italian Holstein-Friesian cows. Livest Prod Sci. 80 (3):211-220.

Shook, G. E. 2006. Major advances in determining appropriate selection goals. J. Dairy Sci. 89(4):1349-1361.

Strandberg, E., and J. Sölkner. 1996. Breeding for longevity and survival in dairy cattle. Proc. Int. Workshop on Genetic Improvement of Functional Traits in

Cattle, Gembloux, Belgium. INTERBULL Bull. No. 12. Int. Bull Eval. Serv., Uppsala, Sweden.

Sun, Y., H.K. Parmentier, K. Frankena, J.J. van der Poel. 2011. Natural antibody isotypes as predictors of survival in laying hens. Poultry Science 90: 2263-2274.

Thompson-Crispi, K.A., Miglior, F., Mallard, B.A. 2013. Genetic parameters for natural antibodies and associations with specific antibody and mastitis in Canadian Holsteins. J. Dairy Sci. 96: 3965-3972.

Thomsen, P. T., and H. Houe. 2006. Dairy cow mortality. A review. Vet. Q. 28:122–129.

Van Knegsel, A. T., G. de Vries Reilingh, S. Meulenberg, H. van den Brand, J. Dijkstra, B. Kemp, and H. K. Parmentier. 2007. Natural antibodies related to energy balance in early lactation dairy cows. J Dairy Sci 90(12):5490-5498.

Van Pelt, M.L., T.H.E. Meuwissen, G. de Jong and R.F. Veerkamp. 2015. Genetic analysis of longevity in Dutch Dairy cattle using random regression. J. Dairy Sci. 98:4117-4130.

VanRaden, P.M., C.P. Van Tassell, G.R. Wiggans, T.S. Sonstegard, R.D. Schnabel, J.F. Taylor, and F. Schenkel. 2009. Invited review: Reliability of genomic predictions for North American Holstein bulls. J. Dairy Sci. 92:16-24.

Zwald, N. R., K. A. Weigel, Y. M. Chang, R. D. Welper, and J. S. Clay. 2006. Genetic analysis of clinical mastitis data from on-farm management software using threshold models. J. Dairy Sci. 89(1):330-336.

2

Phenotypic and genetic relationships of bovine natural antibodies binding keyhole limpet hemocyanin in plasma and milk

B. de Klerk[*], B.J. Ducro[*], H.C.M. Heuven[*,§], I. den Uyl[†], J.A.M. van Arendonk[*], H.K. Parmentier[‡], J.J. van der Poel[*]

[*]Animal Breeding and Genomics Centre, Wageningen University, The Netherlands
[†]Animal Health Services, Deventer, The Netherlands
[‡]Adaptation and Physiology Group, Wageningen University, The Netherlands
[§]Faculty of Veterinary Medicine, Utrecht University, The Netherlands

Journal of Dairy Science 2015 98:1-7

Abstract

To improve the health status (resilience) of dairy cows, levels of natural antibodies (NAbs) might be useful. The objective of the present study was to compare levels, and to estimate genetic parameters for NAbs measured in milk- and plasma samples. Titers of NAb immunoglobulin IgM and IgG isotype binding keyhole limpet hemocyanin (KLH) of 2,919 cows, in both plasma and milk, were measured using ELISA. Analysis revealed that NAb levels in milk significantly increased with parity, while they remained constant in plasma. Moderate positive phenotypic correlations were found between NAb levels in milk and in plasma: 0.18 for IgG and 0.40 for IgM. This indicates that NAbs from milk and plasma might reflect different aspects of a dairy cow's health status. However, high genetic correlations were found for NAbs in milk and plasma: 0.81 for IgG and 0.79 for IgM. Heritabilities for NAbs measured in plasma (0.15 (0.05) for IgG and 0.25 (0.06) for IgM) were higher than heritabilities of NAbs measured in milk (0.08 (0.03) for IgG and 0.23 (0.05) for IgM). Our results indicate that NAbs measured in milk and plasma are heritable and likely have a common genetic background suggesting that NAb levels measured in milk might be useful for genetic improvement of disease resistance.

Keywords: dairy cattle, natural antibody, genetic parameter

2.1 Introduction

Genetic selection for improved disease has become more important because of the limitation of usage of antibiotics. Selection for an animal's health status is a challenging task due to the lack of reliable, cheap and easy to measure parameters. Natural antibodies (NAbs) might be a novel parameter that enables selection of cows with an improved ability to stay healthy and to remain productive over a longer period of time.

In mammals, NAbs represent an important component of innate immunity, forming a first line of defence and linking innate and specific immunity (Ochsenbein and Zinkernagel, 2000). NAbs are defined as immunoglobulins derived from self-renewing CD5+ B-1 cells (Casali and Notkins, 1989; Baumgarth et al., 2005). NAbs are found in all animals tested so far without intentional antigenic stimulation (Avrameas et al., 1991; Ochsenbein and Zinkernagel, 2000) and have been proposed to reflect the ability of an animal to stay healthy and prolong survival (Boes et al., 2000; Star et al., 2007).

NAbs are suggested to play an essential role in induction of a primary immune response and improve immune responsiveness to protect individuals against viral and bacterial pathogens (Kohler et al., 2003, Ochsenbein et al., 1999; Thornton et al., 1994; Lammers et al., 2004). In chicken, NAbs binding keyhole limpet hemocyanin (KLH) were indicative for a higher probability of survival during the laying period (Star et al., 2007; Sun et al., 2011). In cattle, NAbs were found in both plasma and milk (Van Knegsel et al., 2007; Ploegaert et al., 2011) and NAb levels increase with age (Srinivasan et al., 1999; Van Knegsel et al., 2007). A positive phenotypic relationship was suggested in dairy cattle between NAbs measured in plasma and their energy balance, milk yield and dry matter intake (Van Knegsel et al., 2007). However, in milk negative relations were found between NAb levels and energy balance, milk yield and dry matter intake (Van Knegsel et al., 2007). Furthermore, Ploegaert et al. (2010a) suggested that NAbs (especially isotype IgG1) protect against clinical mastitis and high SCC. Heritabilities of KLH binding NAb levels measured in plasma, were 0.18 for IgM and 0.32 for IgG (Thompson-Crispi et al., 2013). Heritabilities of KLH NAbs measured in milk were 0.32 for IgG and 0.41 for IgM (Wijga et al., 2013). Together these results show that NAbs give information on the health perspective of animals and might also be used for genetic selection to improve health traits.

Measurement of NAb levels in milk, rather than in plasma, is non-invasive and cheaper. NAbs measured in milk may thus be a promising parameter reflecting innate immunity, that might be predictive for disease resistance of dairy cows. As a

first step in determining the value of NAbs measured in milk as a parameter of disease resistance, phenotypic and genetic relationships of NAbs measured in plasma and milk is useful. Knowledge of this relationship is currently lacking. The objective of this study is to estimate phenotypic and genetic parameters for NAbs measured in plasma and milk. Genetic parameters will reveal whether NAb levels in plasma and in milk reflect the same trait.

2.2 Material and methods

2.2.1 Animals and Samples

Data used are from the Dutch project "WeerbaarVee" (resilient livestock). In February and March 2011 plasma and milk samples of 3,034 dairy cows were simultaneously collected from 29 Dutch dairy farms spread over the country. Animals with an unknown ID-number or with missing values were excluded from the dataset, so 2,919 cows were actually used for final analyses. To ensure that the results would be representative of future farming conditions, farms were required to have at least 60 cows in milk recording and farms with a mean production level in the lowest quartile were excluded. The farms were chosen from the group of farms with either a high herd age (mean= 4.73 yrs; farm type 1) or an average herd age (mean= 4.16 yrs; farm type 2) in the year prior to the start of this study. All selected farms needed to participate in the national milk production registration system (MPR) and were required to agree to sampling of plasma and milk from cows and detailed disease recording. Most (72,5%) of the cows included in the study were purebred Holstein Friesians. Furthermore, preliminary results showed no significant effect of breed and it was therefore left out for further analyses. Milk samples were collected in conjunction with MPR from all lactating cows. Plasma samples were taken from about 70 cows per farm; both lactating and non-lactating (dry cows and heifers before calving). The cows for collecting plasma samples were selected based on parity and lactation-stage with the aim of keeping the distribution of cows over these different groups as well as possible. Clinically sick animals were not included in MPR and therefore have no milk samples. In total 2,610 milk samples and 2,032 plasma samples were collected. The pedigree file for estimating genetic parameters was provided by CRV (Cooperative cattle improvement organization, Arnhem, The Netherlands) and included 30,436 animals. Cows used within this study originated from 994 sires and 2,367 dams.

2.2.2 Natural Antibodies

NAb isotypes IgM and IgG binding *Megathura crenulata*-derived keyhole limpet hemocyanin (KLH) were determined in individual plasma and milk samples by an indirect two-step ELISA. Flat-bottomed 96-well medium binding plates were coated overnight at 4ºC with 1 µg/ml KLH (MP Biomedicals Inc., Aurora, Ohio) 100 µL/well, in coating buffer (5.3 g/L Na_2CO_3, and 4.2 g/L $NaHCO_3$, pH 9.6). After washing, plasma and milk samples were diluted in 4 steps, with a dilution buffer (phosphate buffered saline (PBS); 10.26 g/L $Na_2HPO_4 \cdot H_2O$, 2.36 g/L KH_2PO_4, and 4.50 g/L NaCl, pH 7.2, containing 0.05% Tween® 20 and 0.5% normal horse serum). Plates were incubated for 1.5 hrs at room temperature (RT) with the samples 1:3 diluted (1:30, 1:90, 1:270, and 1:810). Binding of the antibodies to KLH was visualized using a 1:20.000 diluted rabbit anti-bovine IgGFc labelled with peroxidase (PO) (RABo/IgGFc/PO, Nordic, Tilburg, The Netherlands) and 1:20.000 diluted rabbit anti-bovine IgM (RABo/IgM/PONordic). After washing, substrate (tetramethylbenzidine and 0.05% H_2O_2) was added. After10 min, the reaction was stopped with 2.5 *N* H_2SO_4. Extinctions were measured with a Multiskan (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Antibody levels (titers) were expressed as log2 values of the highest dilutions giving an extinction closest to 50% of EMAX, where EMAX represents the highest mean extinction of a standard positive reaction present on each flat-bottomed ELISA-plate (Ploegaert et al. 2010b). EMAX was calculated based on a plasma calibrated line for each ELISA plate, for both milk and plasma samples. An amount of 3 titer units was added to all obtained NAb titers to create positive values.

2.2.3 Statistical Analyses

Descriptive statistics were calculated using SAS (version 9.2). Variance components for levels of different NAb isotypes in milk and plasma samples were estimated with a linear animal model:

$$Y_{ijklm} = \mu + PAR_i + LAC_j + HERD_k + animal_l + e_{ijkl} \quad (1)$$

where Y were NAb levels (i.e. M-IgG= IgG in milk, M-IgM= IgM in milk, P-IgG= IgG in plasma and P-IgM= IgM in plasma), µ is the population mean, PAR is fixed effect of parity (i=class of parities, where 1 = heifers before first calving, 2= parity 1, 3=parity 2, 4=parity 3, and 5= parity ≥ 4), LAC is fixed effect of lactation stage (where 1=<60 DIM, 2=60-120 DIM, 3=120-200 DIM, 4=200-305 DIM, 5>305 DIM, and 6 = non-

lactating cows), HERD is random herd effect, animal is random additive genetic effect of the cow and e is random residual. Genetic analyses were performed with *ASREML*-software (version 3.0, Gilmour et al., 2009). The estimated variance components from single trait analysis were used to estimate heritabilities. Additionally, phenotypic and additive genetic correlations were calculated from variance components obtained by bivariate analysis.

2.3 Results

2.3.1 Descriptive Statistics

Preliminarily analysis of the data revealed that all NAb titers were normally distributed. Means and standard deviations of all four NAbs binding KLH (IgM and IgG in both plasma and milk) are shown in Table 2.1. Mean levels of both IgG and IgM in milk were lower than those detected in plasma. The trait with most variation was IgG in plasma.

In total 29 farms participated in this study ranging between 53 and 189 lactating cows per farm, with a mean of 89 lactating cows. Mean herd age of productive cows on farms participating in the study was 4.8 (4.3-6.0) years. Generally farms classified as 'high' had lower NAb levels than farms classified as "average", for both NAb levels in milk and plasma. Nevertheless, no significant differences in NAb levels were found between these two groups. Farm-type was therefore not included in further analyses.

Table 2.1. Numbers, means and standard deviations (SD) for titers of natural antibody isotypes (IgG and IgM) measured in both milk (M) and plasma (P) samples.

Antibody[1]	N	Mean	SD
M-IgG	2,610	3.25	0.83
M-IgM	2,610	5.60	1.07
P-IgG	2,032	8.03	1.24
P-IgM	2,032	11.56	0.88

[1] Name of NAb where M-IgM = IgM in milk, M-IgG = IgG in milk, P-IgM = IgM in plasma and P-IgG = IgG in plasma

The cows in this study (n=2,919) had a mean parity of 2.74 (max =14) and were on average 205 days in milk (max = 877). Plasma samples were taken from 3 groups:

lactating cows, dry cows and pregnant heifers. Higher NAb levels in milk and blood were detected in dry as compared to lactating cows (Table 2.2). We found that parity significantly ($P<0.001$) affected NAb levels in milk: Overall, NAb levels in milk increased with parity (Table 2.2). NAb levels in plasma were not significantly influenced by parity (Table 2.2). IgM levels in milk significantly decreased after the first lactation stage, and significantly increased again towards the end of the lactation period. The same patterns for lactation stages were also observed for IgG, except between the first 3 lactation stages, no significant differences were found. NAb levels in plasma only slightly raised with increasing lactation stage (Table 2.2).

2.3.2 Genetic and Phenotypic Parameters

Estimates for variance components and heritabilities were obtained from a linear animal model, and are presented in Table 2.3. Heritability estimates ranged between 0.08 (SE = 0.03) and 0.25 (SE = 0.06). IgM binding KLH had higher heritability estimates for both milk and plasma (0.23 and 0.25) compared to IgG binding KLH (0.08 and 0.15). Heritabilities of NAb levels were higher in plasma for both IgG and IgM (0.15 and 0.25) than in milk (0.08 and 0.23). Positive phenotypic and genetic correlations between NAb levels measured in plasma and milk for both IgG and IgM (Table 2.4), were observed. Phenotypic correlations between the same isotype in plasma and milk were moderate for both IgM and IgG binding KLH (0.40 and 0.18 respectively). Genetic correlations were higher than phenotypic correlations. We found high positive genetic correlations between the same isotypes in plasma and milk (0.79 for IgM and 0.81 for IgG).

Table 2.2. Corrected means (LSMEANS) for NAb levels in both milk and plasma for different parities (PAR-Class) and different lactation stages (LAC-class).

		M-IgG[1]	M-IgM[1]	P-IgG[1]	P-IgM[1]
LAC-Class[2]	1	3.11[a]	5.39[a]	7.96[a]	11.36[a]
	2	3.04[a]	5.14[b]	8.25[a]	11.61[b]
	3	3.11[a]	5.31[a]	8.17[a]	11.48[ab]
	4	3.31[b]	5.81[c]	8.09[a]	11.55[ab]
	5	3.59[bc]	6.30[d]	8.09[a]	11.62[b]
	6 (dry cows)	X	X	8.25[a]	11.99[c]
PAR-Class[3]	1 (heifers before calving)	X	X	8.58[a]	11.60[a]
	2	3.29[a]	5.52[a]	8.08[a]	11.56[a]
	3	3.33[ac]	5.78[b]	7.93[ab]	11.67[a]
	4	3.41[bc]	5.99[c]	7.91[ab]	11.62[a]
	5	3.45[b]	5.96[c]	7.77[b]	11.51[a]

[abcd] Least square means that do not share a superscript are significantly different (P<0.05)
[1] Name of antibody is antibody isotype (IgG/IgM) combined with milk (M) or plasma (P) sample
[2] Lactation stage classes 1-6, where 1=<60 DIM, 2=60-120 DIM, 3=120-200 DIM, 4=200-305 DIM, 5>305
DIM, and 6 = non-lactating cows
[3] Parity classes 1-5 where 1 = heifers before first calving, 2= parity 1, 3=parity 2, 4=parity 3, and 5= parity ≥ 4

Table 2.3. Estimates and corresponding standard errors of variance components and heritability NAb isotypes IgG and IgM in both milk and plasma[1].

Antibody	σ_a^2	σ_h^2	h^2 (SE)
M-IgG	0.05	0.07	0.08 (0.03)
M-IgM	0.22	0.04	0.23 (0.05)
P-IgG	0.22	0.15	0.15 (0.05)
P-IgM	0.19	0.07	0.25 (0.06)

[1] $h^2 = \sigma_a^2 / \sigma_p^2$; where σ_a^2 was the additive genetic variance, σ_p^2 was the phenotypic variance ($\sigma_a^2+\sigma_h^2 + \sigma_e^2$), with σ_e^2 as the environmental variance and σ_h^2 as the herd variance.

Table 2.4: Phenotypic (above diagonal) and genetic (below diagonal) correlations with standard errors shown in parentheses, between IgG and IgM measured in both plasma and in milk.

	M-IgM[1]	M-IgG[1]	P-IgM[1]	P-IgG[1]
M-IgM	-	0.48 (0.06)	0.40 (0.05)	0.16 (0.04)
M-IgG	0.64 (0.17)	-	0.13 (0.04)	0.18 (0.02)
P-IgM	0.79 (0.09)	0.14 (0.23)	-	0.34 (0.04)
P-IgG	0.29 (0.20)	0.81 (0.18)	0.09 (0.20)	-

[1] Name of NAb where M-IgM = IgM in milk, M-IgG = IgG in milk, P-IgM = IgM in plasma and P-IgG = IgG in plasma

2.4 Discussion

The aim of this study was to provide greater insight on the levels of NAb isotypes binding KLH collected on the same day both in plasma and milk. A genetic analysis was performed on NAb levels in a large group of cows. Genetic and phenotypic correlations were estimated between NAb levels measured in plasma and milk.

2.4.1 Use of Samples

Different isotypes of NAbs can be measured. A large proportion of NAbs in circulation is expected to be of the IgM isotype. Therefore, in this study IgM isotype was studied. In addition to IgM, IgG was also chosen since these two isotypes differ from each other in both physiological function and structure. IgG has a monomeric structure whereas IgM has a pentameric structure. Isotype IgM is polyreactive and

more involved in early, innate immune responses, while IgG plays a role in the adaptive response (Schroeder & Cavacini, 2010). Therefore analysing these two isotypes is expected to provide more insight in potential differences in NAb levels in plasma and milk. Although IgA can be found in milk, the levels of IgA in plasma were below the detection limit of the assay used for this study, and were therefore not included in present analysis.

In the present study, NAbs binding KLH were used. Other studies on NAbs in dairy cows included NAbs binding innate antigens such as lipolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglycan (PGN) (Knegsel et al., 2007, Ploegaert et al. 2010b and 2011, Wijga et al. 2013). These other antigens are present on bacteria which are common in the environment of most dairy cows. KLH, on the other hand, is likely not present in the environment. Exposure to the innate antigens before sampling cannot be excluded, which may complicate comparison of our results with results from other research with other antigens.

NAb levels are influenced by different environmental factors. In the present study, mean NAb levels varied between farms. This is in accordance with earlier studies (Ploegaert et al., 2010b; Wijga et al., 2013). This may be because of housing facilities, farm management and feeding, or genetic factors resulting from differences in breeding decisions over time.

In the present study, no specific groups of animals were chosen. Within other studies like Ploegaert et al. (2011), only cows with low SCC were used; Ploegaert et al. (2010b) and Wijga et al. (2013) only used 1st parity cows. Our analysis revealed a clear impact of parity on levels of NAb binding KLH in milk which confirms earlier findings in dairy cows by van Knegsel et al. (2007). This implies that also the age composition of a group of cows should be considered when comparing genetic parameters between studies. This could be explained by the fact that younger animals are less exposed to exogenous stimuli than older animals. For example, heritabilities might be lower in a more diverse group of animals, with respect to age, due to more environmental variation. However, other studies in poultry (Berghof et al. 2010) and mammals showed that NAb levels do not only depend on exogenous stimuli, but are also related to auto-antigens (Cheng and Chamley, 2008).

2.4.2 Phenotypic parameters of NAb levels in milk and plasma

The pentameric structure of IgM results in a higher binding (avidity) to antigens than IgG, which has a monomeric structure. Secondly, IgM is more abundantly present in the vertebrates body than IgG (Ehrenstein and Notley 2010). IgM is the

first antibody isotype that is produced by B cells. In reaction to antigenic challenge, IgM producing B-cells can switch to B-cells producing other isotypes under the influence of activated by T-cells (Market and Papavasiliou, 2003). In the present study, levels of IgG binding KLH showed more variation than IgM in plasma (Table 2.1). IgG levels are likely to be more affected by environmental exposure to bacteria and other antigens. Wijga et al. (2013) and Thompson-Crispi et al. (2013) found similar results, in milk and plasma respectively.

The present findings showed that NAb levels were lower in milk than in plasma, this is in agreement with earlier studies (Ploegaert et al., 2011, van Knegsel et al. 2007) NAb isotypes IgM and IgG may both be transported from plasma to milk (Kacskovics et al., 2000; Östensson & Lun, 2008) in varying degrees depending on stage of lactation, colostrogenesis, and also in response to inflammation in the mammary gland. Phenotypic correlations between NAb levels measured in plasma and milk were moderately positive (Table 2.4), which means that these correlations are not close to one and are lower than genetic correlations. This can be due to noise in measurements, but also might indicate that NAbs measured in milk and plasma might reveal different information regarding the health status of a cow. This finding suggests that phenotypically NAb levels in milk do not perfectly mirror NAb levels in plasma. The positive and high genetic correlations found in this study (Table 2.4) support a common background of NAb levels in milk and plasma. In an earlier study on 17 cows (different parities and lactation stages, but only cows with a low SCC) a higher positive phenotypic correlation (0.69) was found between the average of 10 individual measurements of total NAb levels binding KLH in milk and plasma (Ploegaert et al. 2011). Using an average rather than single observation reduces the impact of measurement errors and consequently will lead to higher correlations. In contrast to our results, Knegsel et al. (2007) found a small but negative phenotypic correlation between NAb levels in plasma and milk (-0.10). In the latter study, only 76 first parity cows during early lactation were used. We showed, that cows during first stages of lactation have different NAb levels than in later stages of lactation. Such differences in results may rest not only in number of cows but also on distribution over stage of lactation and parity.

2.4.3 Genetic background of NAb levels in milk and plasma

High positive genetic correlations between level of NAb binding KLH measured in plasma and milk for both IgG and IgM were found (0.79 and 0.81 respectively). This suggests that IgM and IgG measured in plasma and milk are regulated by the same (group of) genes. To our knowledge, no other studies published genetic

correlations between NAb levels in plasma and milk. Additionally, we found a moderately positive (0.48) genetic correlation between IgG and IgM binding KLH measured in milk. Wijga et al. (2013) found a small but negative genetic correlation between IgM and IgG binding KLH (-0.10) in milk. Thompson-Crispi et al. (2013) found a negative genetic correlation (-0.41) between IgM and IgG binding KLH in plasma, while in the present study a small but positive genetic correlation for both isotypes binding KLH was found.

The present study confirmed that a substantial part of variation between cows of NAb levels binding KLH in both milk and plasma is heritable (Table 2.3). Heritabilties of IgM and IgG binding KLH in milk and plasma were estimated to be between 0.08 and 0.25. Heritabilities of NAb levels in plasma were higher than heritabilities of NAb levels in milk (Table 2.3). IgM had higher heritabilities for both milk and plasma. This is in agreement with findings of Wijga et al. (2013) who also found the higher heritability (across herd) for IgM (0.41 (SE=0.09)) and a lower heritiablity for IgG (0.32 (SE=0.08)) binding KLH in milk. Higher estimates of heritabilities for IgM might be explained by the fact that IgM is naturally present and more responsible for the first line defence regardless of environmental factors (Kohler et al. 2003, Ehrenstein et al. 2010), where IgG is more influenced by environmental factors, which therefore could explain the lower heritabilities for IgG. However, the heritiabilities of Wijga et al. (2013) are almost doubled, this can be explained by the use of different datasets. Wijga et al. (2013) used 1695 first parity cows from 380 farms sired by 100 bulls. Additionally, only cows off at least 87.5% Holstein Friesian were included. Therefore, cows will have less genetic variation what may cause differences compared to present results. Ploegaert et al. (2010b) found higher heritabilities of NAbs binding KLH in milk (0.36 (across herds) and 0.42 (intra herd)), but no distinction was made between NAb isotypes. Thompson-Crispi et al. (2013) found a higher heritability for IgG (0.32) than the heritability of IgM (0.18) both binding KLH in plasma, which is opposite to current findings. In that study, however, cows were immunized with hen egg white lysozyme before sampling which is known to stimulate the antibody mediated immune response (AMIR). Therefore, this immunization of cows makes it hard to compare their results to our study which is directed to naturally present antibodies binding KLH.

From our findings it can be concluded that NAbs in both milk and plasma share a common genetic background and show potential for genetic selection. Selection of cows based on NAb levels measured in milk will also lead to correlated response of NAbs in plasma. Measurements of NAb levels in milk are less invasive and cheaper than measurements in plasma. Measurement of NAb levels in milk is a more feasible option for genetic evaluation of sires based on a high number of offspring.

The value of selection on parameters for NAb depends on its relationship with a cows' health status and longevity of dairy cows which is currently being investigated.

2.5 Conclusion

Simultaneously measured NAb levels in both plasma and milk showed high positive genetic correlations for both IgM and IgG binding KLH. This indicates that they are regulated by the same set of gene(s). However, phenotypically NAb levels might give different information on different aspects of a cow's immune status, inferred from to lower phenotypic correlations. NAb isotypes IgG and IgM binding KLH in both plasma and milk are heritable, where IgM is more heritable than IgG. Our results indicate that NAbs may be valuable for designing breeding strategies for improved health or resilience in cows. Information on NAbs as selection tool can be obtained from milk samples, since genotypic correlations with NAbs measured in plasma are high. Further studies are in progress to unravel the relations between NAb levels and diseases and to acquire detailed information on reasons for variability in NAb levels influenced by factors like farm management, genetic background and functional differences between milk- and plasma NAbs.

2.6 Acknowledgements

This study is part of a joint project WeerbaarVee, which is funded by the Dutch Ministry Economic Affairs (The Hague, the Netherlands), Productschap Zuivel (Dutch Dairy Product Board, Zoetermeer, the Netherlands), CRV (Arnhem, the Netherlands), LTO Noord Fondsen (Zwolle, the Netherlands), Animal Health Services (Deventer, the Netherlands), and Wageningen University (Wageningen, the Netherlands). The authors also thank all the herd owners for their collaboration and help with collecting data.

2.7 References

Avrameas, S. 1991. Natural autoantibodies: from ' horror autotoxicus' to ' gnothu seauton'. Immunol. Today 12, 154-159.

Baumgarth, N., Tung, J.W., Herzenberg, L.A. 2005. Inherent specifities in natural antibodies: a key to immune defence against pathogen invasion. Springer Semin Immunopathol. 26: 347-362.

Berghof, T.V.L., De Vries Reilingh, G., Nieuwland, M.G.B., Parmentier, H.K. 2010. Effect of aging and repeated intratracheal challenge on levels of cryptic and overt natural antibodies in poultry. Poult. Sci. 89:227-237.

Boes, M. 2000. Role of natural and immune IgM antibodies in immune responses. Mol. Immunol. 37: 1141-1149.

Casali, P. and Notkins, A.L. 1989. CD5+ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. Immunol. Today 10, 364-368.

Cheng, H.M, Chamley, L. 2008. Cryptic natural antibodies and co-potentiators. Autoimmunity 7 (2008) 431-434.

Ehrenstein, M.R., and Notely, C.A. 2010. The importance of natural IgM; scavenger, protector and regulator. Nature Rev. Immunol. 10, 778-786.

Gilmour, A.R., Cullis, B.R., Thompson, R. 2009. *ASREML* User Guide. Release 3.0. VSN International Ltd., Hemel Hempstead, UK.

Kacskovics, I., Wu, Z., Simister, N.E., Frenyó, L.V., Hammerström, L. 2000. Cloning and characterization of the bovine MHC class I-like Fc receptor. J. Immunol. 2000, 164:1889-1897.

Kohler, H., Bayry, J., Nicoletti, A., Kaveri, S.V. 2003. Natural antibodies as tools to predict the outcome of immune response?. Scand. J. Immunol. 58: 285-289.

Lammers, A., Klomp, M.EV., Nieuwland, M.G.B, Savelkoul, H.F.J., Parmentier, H.K., 2004. Adoptive transfer of natural antibodies to non-immunized chickens affects subsequent antigen-specific humoral and cellular immune responses. Dev. Comp. Immunol. 28, 51-60.

Market, E., and Papavasiliou, F.N., 2003. V(D)J Recombination and the evolution of the adaptive immune system. PLoS Biol. 1, 24-27.

Ochsenbein, A.F., Fehr, T., Lutz, C., Suter, M., Brombacher, F., Hengartner, H., Zinkernagel, R.M. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. Science 286, 2156-2159.

Ochsenbein, A.F., Zinkernagel, R.M. 2000. Natural antibodies and complement link innate and acquired immunity. Immunol. Today, December 2000; vol. 21, no. 12:624.

Östensson, K. and Lun, S. 2008. Transfer of immunoglobulins through the mammary endothelium and epithelium and in the local lymph node of cows during the initial response after intramammary challenge wit *E. coli* endotoxin. Acta. Vet. Scand. 50(1), 26.

Ploegaert, T.C.W. 2010a. Relation of natural antibodies with risk for mastitis and high somatic cell count in Dutch Holstein Friesian cows. In: Parameters for natural resistance in bovine milk (Phd Thesis), p 82-92. Wageningen University, Wageningen, The Netherlands. ISBN: 978-908585-827-0.

Ploegaert, T.C.W., Wijga, S., Tijhaar, E., van der Poel, J.J., Lam, T.J.G.M., Savelkoul, H.F.J., Parmentier, H.K., van Arendonk, J.A.M. 2010b. Genetic variation of natural antibodies in milk of Dutch Holstein-Friesian cows. J. Dairy Sci. 93: 5467-5473.

Ploegaert, T.C.W., Tijhaar, E., Lam, T.J.G.M., Taverne-Thiele, A., van der Poel, J.J., van Arendonk, J.A.M., Savelkoul, H.F.J, Parmentier, H.K. 2011. Natural antibodies in bovine milk and plasma plasma: Variability among cows, repeatability within cows, and relation between milk and plasma titers. Vet. Immunol. Immunopathol. 144, 88-94.

Schroeder, H.W., & Cavacini, L. 2010. Structure and function of immunoglobulins. J Allergy Clin. Immunol. 125, S41-52.

Srinivasan, A., NI, Y., Tizard, I. 1999. Specifity and prevalence of natural bovine antimannan antibodies. Clin. Diagn. Lab. Immunol. 6 (6):946.

Star, L., Frankena, K., Kemp, B., Nieuwland, M.G.B., Parmentier, H.K. 2007. Natural Humoral Immune Competence and Survival in layers. Poult. Sci., 86: 1090-1099.

Sun, Y., Parmentier, H.K., Frankena, K., van der Poel, J.J. 2011. Natural antibodies isotypes as predictors of survival in laying hens. Poult. Sci. 90:2263-2274.

Thompson-Crispi, K.A., Miglior, F., Mallard, B.A., 2013. Genetic parameters for natural antibodies and associations with specific antibody and mastitis in Canadian Holsteins. J. Dairy Sci. 96: 3965-3972.

Thornton, B.P., Vetvicka, V., Ross, G.D., 1994. Natural antibody and complement-mediated antigen processing and presentation by B lymphocytes. J. Immunol. 152, 1727-1737.

Van Knegsel, A.T.M, de Vries Reilingh, G., Meulenberg, S., van den Brand, H., Dijkstra, J., Kemp, B., Parmentier, H.K. 2007. Natural antibodies related to energy balance in early lactation dairy cows. J. Dairy Sci. 90: 5490-5498.

Wijga, S., Bovenhuis, H., Bastiaansen, J.W.M., van Arendonk, J.A.M., Ploegaert, T.C.W., Tijhaar, E., van Poel, J.J. 201 3. Genetic parameters for natural antibody isotype titers in milk of Dutch Holstein-Friesians. Anim. Genet. 44: 485-492.

3

Relation between antibody levels in milk and productive lifetime for Dutch dairy cows

B. de Klerk[*], B.J. Ducro[*], H.C.M. Heuven[†,*], J.A.M. van Arendonk[*], H.K. Parmentier[‡], J.J. van der Poel[*]

[*]Animal Breeding and Genomics Centre, Wageningen University, Wageningen, The Netherlands, P.O. Box 338
[†] Genetwister Technologies B.V, Wageningen, The Netherlands, P.O. Box 193
[‡] Adaptation Physiology Group, Wageningen University, Wageningen, The Netherlands, P.O. Box 338193

Submitted to Journal of Animal Breeding and Genetics

Abstract

In dairy cattle, selection for longevity is often used to improve profitability. However, a cow's longevity becomes available only after culling, which slows the selection progress. Longevity is often expressed as productive lifetime (PL), i.e. time between first calving and actual death of a cow. Antibodies (Ab) are an important component of the cow's immune system and health status, and can be measured during early life. PL is related at least in part with health, therefore, antibodies might be a useful predictor for PL. First objective, was to examine if Ab (levels and isotypes) measured in milk of heifers, predict length of a cow's PL. Second objective was to study the added value of Ab (isotype) levels as a predictor of PL compared to one of the currently used predictor of PL, somatic cell score (SCS). Levels of three Ab isotypes (IgG1, IgA and IgM) in 1515 heifers, were measured, binding four antigens: lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN), respectively, and presumably natural antibodies (NAb) binding keyhole limpet hemocyanin (KLH). Each Ab binding the four antigens, showed the same pattern as SCS; an increase in Ab was associated with a shorter PL. Also, it was concluded that Ab can give additional information on the length of PL, besides information given by SCS.

Keywords: Dairy cattle, immunity, antibody, longevity

3.1 Introduction

Early disposal of dairy cows can partly be prevented by selection for longevity. Longevity reflects a cow's ability to survive instead of being culled (Vollema, 1998). Longevity can be defined as the productive lifetime (PL) of a cow, referring to the time (in days) between first calving and the last test date of a cow. Productive lifetime is closely related to profitability of a cow's milk production as a longer PL allows a cow to reach its maximum production, reduces the losses due to involuntary culling and reduces costs of replacement (Rogers et al., 1988). Thus, it is economically beneficial to extend PL by decreasing involuntary culling. However, PL has low heritability (van Pelt et al. 2015), and can only be measured after a cow is culled. This takes time and goes at the expense of effective selection. Given the economic importance but late availability of PL, there is a demand for suitable alternative prediction parameters. Currently, one of the generally accepted alternative methods is using somatic cell count (SCC) or somatic cell score (SCS) as predictors in survival analysis. SCS is considered as an indirect measure of the inflammatory status of the udder, or mastitis, as well as a milk quality parameter (Samoré et al., 2003). Strong positive correlations were reported between SCS and culling rate, and therefore influence PL in dairy cows (Samoré et al., 2003, Sewalem et al., 2006). One advantage of using SCS as predictors for PL, instead of using PL directly, is that SCS can be measured in early life (as soon as first lactation starts). Moreover, measurements of SCS from routinely collected milk samples have become a general standard for dairy industry, thus no extra time or money is needed to obtain these measurements. Despite the above mentioned advantages, SCS is not a perfect predictor. Information obtained from SCS mainly focusses on PL associated with udder-health status. However, PL is not only affected by udder-health status. The question, therefore, arises: is there another, more general not-tissue, but immune-related parameter which can be measured easily in milk, early in life and is predictive for the length of PL? Antibodies (Ab) in serum or milk might be an option. Ab can be divided in different groups: specific antibodies (SpAb) produced in human and mice by B-2 B cells, raised antigen-specifically, as part of the adaptive immune system (Baumgarth, 2011), and albeit less well known, natural antibodies (NAb), in man and mice produced by B-1 B cells, that were shown to provide a more immediate, early and broad first line of defence against pathogens in a nonspecific fashion, making them an important component of the naive immune system (Baumgarth et al., 2005). Though little is still known of the mechanisms underlying the production of NAb, they are produced at tightly regulated levels in the absence of external antigenic stimulation (Dunkelberger and

Song, 2010). It is reasonable to suggest that Ab, especially in blood, but likely also in milk may be more informative of the animal's immune and general health status compared to SCS, since SCS is associated with local udder-health. Relations between Ab (SpAb and NAb) and diseases have been studied before. With respect to SpAb, cows with a higher antibody mediated immune response (AMIR) showed lower incidence of mastitis (Thompson-Crispi et al. 2013b,). Regarding to NAb, Ploegaert et al. (2010b) reported a possible protective role of certain isotypes of NAb in milk preventing clinical mastitis and high SCC in healthy heifers. Van Knegsel et al. (2012) found that levels of NAb binding KLH or lipopolysaccharide (LPS) measured in plasma and in milk in early lactation are related to the metabolic health status of dairy cows. And recently, Mayasari et al. (submitted) proposed that Ab binding KLH or LPS, respectively, were related with enhanced risk of mastitis or enhanced SCC. This suggests that levels (and isotypes of) Ab can be used as a health indicator (Ploegaert et al., 2011, Van Knegsel et al., 2012). In chickens, Star et al. (2007) and Sun et al. (2011) showed that total plasma levels of NAb binding KLH, but also the plasma levels of IgM and IgG isotypes were indicative for the probability of chickens to survive their laying period. To our knowledge, in dairy cattle so far no relations between Ab and PL have been reported.

Productive lifetime is often hard to implement within breeding programs, since information on this trait is only available at the end of an animal's life. If Ab levels, measured in early life, such as the first lactation, are correlated with PL, they can act as an early predictor for PL. It is, therefore, important to determine the relation between Ab and length of PL. This relation largely determines the accuracy of selection for PL that can be achieved based on measurements of Ab at an early age. The objectives of this study were therefore, (1) to investigate whether Ab of different isotypes (IgG1, IgA and IgM) binding different antigens (LPS, lipoteichoic acid (LTA), peptidoglycan (PGN) and KLH, measured in milk of heifers are predictive for the length of a cow's PL (where Ab binding the naive antigen KLH, are assumed to be NAb), (2) to study the added value of Ab as a predictor of PL in addition to SCC.

3.2 Material and Methods

3.2.1 Animals

Data were collected as part of the Dutch Milk Genomics Initiative. Ab levels were determined in milk samples collected from 1515 dairy cows, as described by

Ploegaert (2010b). Cows originated from 339 commercial dairy herds in the Netherlands. Each herd contributed between one to ten cows in this study. All milk samples were collected between Feb 9th and May 3rd in 2005. After deleting cows with missing values for either culling dates or Ab levels, 1455 cows were included in the final data-set, which were all culled between the 1st of April 2005 and the 20th of December 2012. The included heifers were between 66 days and 282 days in lactation, with a mean of 167 days. A majority of the cows calved in the summer (June-August, 2004) and autumn (September-November, 2004), with the percentage of 35.8% and 61.7% respectively. Only around 2.5% of cows calved in winter (December – February, 2004). All cows first calved between the age of 656 days and 1086 days, with an average of 777 days. Culling dates of cows and SCC of milk samples were retrieved from the database of the cattle cooperative CRV (Arnhem, The Netherlands). PL was calculated as the difference between the date of first calving and the date of culling. SCS from milk samples was calculated as the natural logarithm of SCC/1000.

3.2.2 Antibodies

Levels (titers) of antibodies (Ab) were measured in morning milk samples by indirect ELISA. Plates were coated with 100 µl/well of 1 µg of *Megathura crenulata*-derived keyhole limpet hemocyanin (KLH, MP Biomedicals, Solon, OH), , 4 µg of *Eschericia coli*-derived lipopolysaccharide, (LPS, L2880,serotype O55:B5, Sigma-Aldrich Inc. St. Louis, MO), 5 µg of *Staphylococcus. aureus*-derived lipoteichoic acid (LTA, L2515, Sigma-Aeldrich), or 2 µg of *Staph aureus*-derived peptidoglycan (PGN, Bio-Chemika, Buchs, Switzerland), respectively, per ml of carbonate buffer (10.6 g/L Na2CO3, pH 9.6). After incubation of 24 hours at 4°C, plates were washed and blocked with 100 µl/well of 2.5% rabbit serum in PBS with 0.05% Tween-20 for at least 30 minutes at room temperature (21°C). Four serial dilutions of samples (1:4) in PBS, 0.05% Tween20, and 2.5% rabbit serum were added. Dilutions started at 1:4, and on each plate the same positive control sample was included with 8 serial dilutions (1:2), in duplicate. Plates were incubated for 1 hour at room temperature. Binding of Ab to LTA or LPS, or PGN, or KLH, respectively, was detected using 1:16,000 diluted sheep anti-bovine IgG1 (Serotec, Dusseldorf, Germany), 1:16,000 diluted sheep anti-bovine IgM (Serotec), or 1:8,000 diluted sheep anti-bovine IgA (Serotec), all coupled to horseradish peroxidase. After washing, 100 µL/well of tetramethylbenzidine (71.7 µg/mL) and 0.05% H2O2 were added to the wells and incubated for 10 min at room temperature (21°C). The reaction was stopped with 50 µL/well of 2.5 N H2SO4. Extinctions were measured with a Multiskan

spectrophotometer (Flow, Irvine, UK) at a wavelength of 450 nm. Ab levels were calculated as titers, where titers were expressed as log2 values of the dilutions that gave an extinction closest to 50% of E*max.* E*max* represents the highest mean extinction of a standard positive milk sample present in duplicate on every microtiter plate.

In this study, 98 different plates and 10 columns were randomly selected and used in the process of scaling the Ab titers. As a result, twelve Ab isotype titers binding four different antigens were obtained: Ab isotype IgM binding KLH (IgM-KLH); LPS (IgM-LPS), PGN (IgM-PGN), or LTA (IgM-LTA), Ab isotype IgG1 binding KLH (IgG1-KLH); LPS (IgG1-LPS); PGN (IgG1-PGN); LTA (IgG1-LTA); and Ab isotype IgA binding KLH (IgA-KLH); LPS (IgA-LPS); PGN (IgA-PGN); LTA (IgA-LTA).

3.2.3 Statistical Analyses

Descriptive statistics and outcomes of regression models were obtained using SAS (version 9.2). Every Ab binding to different antigens was pre-adjusted using the following model:

$$Y_{ijkl} = \mu + b_1 \text{ x } DIM_i + b_2 \text{ x } AFC_j + BATCH_k + FARM_l + e_{ijkl} \quad (1)$$

Where the response variable Y represented the different Ab binding different antigens (i.e. IgM-KLH, IgM-LPS, IgM-LTA, IgM-PGN, IgG1-KLH, IgG1-LPS, IgG1-LTA, IgG1-PGN, IgA-KLH, IgA-LPS, IgA-LTA, IgA-PGN), μ was the population mean, DIM was covariate describing the effect of days in milk, with regression coefficient b_1, AFC was covariate describing the effect of age at first calving in days, with regression coeficient b_2, BATCH was fixed effect of day of analysis (where *k* was batch-class with 1=day 1, 2=day 2), FARM was random herd effect and e was the random residual.

For Ab binding to antigen LTA same fixed effects were used, except for 'BATCH', since LTA Ab were tested on 9 days instead of only 2. Therefore, in this case BATCH was fixed effect of day of analysis (where *k* was batch-class with 1=day 1 and 2=day 2, 3=day 3, 4= day 4, 5=day 5, 6=day 6, 7=day 7, 8=day 8 and 9=day 9).

Productive lifetime (PL) was pre-adjusted using the following model:

$$Y_i = \mu + YS_i + FARM + e_i \quad (2)$$

Where the response variable Y represented the productive lifetime (PL) in days, μ was the population mean, YS was the fixed effect of the combination of year (Y)

and season (S) of culling, FARM was the random herd effect and e was the random residual.

For the second aim of this study Ab levels were compared to SCS, SCS was pre-adjusted using model 3:

$$Y_i = \mu + b_1 \times MY_i + FARM_j + e_{ij} \quad (3)$$

Where the response variable Y represented the SCS, μ was the population mean, MY was fixed effect of daily milkyield (kg milk) on day of sampling, FARM was random herd effect and e was the random residual.

Regression analyses using the pre-adjusted variables were subsequently performed to estimate the influence of each Ab on PL.

Furthermore, step-wise regression was applied to investigate if a set of Ab levels can be found that better fit the data. Stepwise regression was performed both including and excluding SCS as one of the eligible variables. This helps to determine the added value of Ab levels to information gained from current parameter SCS. Additionally, predicted difference in days of PL between 10% of animals with lowest Ab levels and 10% of animals with 10% highest Ab levels were estimated.

3.3 Results

Descriptive results from levels of a total of twelve Ab isotype-antigen combinations in milk are shown in Table 3.1. Means of Ab levels directed to LPS, PGN and LTA ranged from 0.65 to 5.47 and means for Ab levels binding KLH ranged from 2.32 to 4.76. Standard deviations ranged from 0.98 for KLH-IgM to 1.80 for LTA-IgM. Isotype IgG1 antibodies binding KLH and LPS showed greatest standard deviation. PGN-IgA showed a slightly greater standard deviation than IgG and IgM. With respect to antigen, LTA; IgM showed greatest standard deviation. SCS varied from 1.10 to 8.77, with a mean of 3.74 and a standard deviation of 1.06.

3.3.1 Productive Lifetime and Antibodies

Productive lifetime (PL) was calculated based on differences in days between day of first calving and day of culling. In this study each of 1515 animals had a known culling date and could therefore be included within the final analysis. The PL ranged from 84 days to 3051 days with a mean of 1244 days and a standard deviation of 678 days. Parities ranged from 1 to 5, where 16.0% of the cows only had one lactation, 23.6% had two lactations, 27.4% had three lactations, 32.6% had four lactations and 0.4% had five lactations. After regression, regarding PL, all Ab

showed the same trend. When the level of Ab increased with 1 point, the decrease in PL of a cow was between approximately 17-50 days (Table 3.2). A similar trend

Table 3.1. Overview of means and standard deviation of all different antibodies (Ab[1]).

Ab[1]	N	Mean	Std Dev
KLH-IgA	1455	2.32	1.19
KLH-IgG1	1452	4.76	1.34
KLH-IgM	1455	2.53	0.98
LPS-IgA	1455	2.41	1.21
LPS-IgG1	1455	2.61	1.50
LPS-IgM	1452	3.15	1.06
PGN-IgA	1455	0.65	1.39
PGN-IgG1	1448	5.47	1.38
PGN-IgM	1455	0.85	1.11
LTA-IgA	1454	4.92	1.00
LTA-IgG1	1455	5.02	1.23
LTA-IgM	1455	4.92	1.80

[1]Name of antibody/antigen combination. Where KLH = keyhole limpet hemocyanin, LPS=lipopolysaccharide, LTA = lipoteichoic acid, PGN = peptidoglycan, IgA = Ab isotype IgA, IgG1 is Ab isotype IgG1 and IgM is Ab isotype IgM.

was seen for SCS, if SCS increased with 1 point, the PL of a cow decreased with approximately 62 days. In the case of LPS-IgA, PGN-IgG1, LTA-IgA, LTA-IgM and SCS these relationships with PL were significant (Table 3.2). The predicted difference in days of PL between the 10% lowest Ab level animals and the 10% highest Ab level animals was estimated (Table 3.2). With a mean PL of 1244 days, we observed a predicted difference of PL between approximately 47 and 198 days. For SCS the predicted difference between the 10% lowest Ab level animal and 10% highest Ab level animals was 201 days.

3.3.2 SCS and Antibodies vs Productive Lifetime

After a stepwise regression procedure the combination of SCS and Ab that best explained variation in length of PL was estimated. The level of PGN-IgG1 together with SCS explained greatest variation of PL (Table 3.3). Adding another Ab did not significantly improve the prediction of PL. When SCS was not included in the stepwise procedure, the best combination to predict PL was levels of PGN-IgG1

with levels of LTA-IgA (Table 3.3). Combining SCS and levels of PGN-IgG1 resulted in a predicted difference of approximately 224 days between the 10% lowest Ab level animals and the 10% highest Ab level animals. When SCS was not included in the stepwise regression procedure, the combination of PGN-IgG1 and LTA-IgA levels resulted in a predicted difference of PL of approximately 207 days between the 10% lowest Ab level animals and the 10% highest Ab level animals.

Table 3.2. Regression coefficients between antibodies (Ab[1]) and productive lifetime (PL) in days with standard errors, p-values, R^2 and prediction of differences in days between 10% highest en 10% lowest animals for Ab and SCS (pred_contrast).

Ab	Regression coefficient	s.e.	p-value	R^2	Pred_ contrast
KLH-IgG1	-22.48	16.21	0.166	0.0013	85.80
KLH-IgA	-30.64	18.64	0.101	0.0019	101.72
KLH-IgM	-16.06	21.28	0.451	0.0004	46.83
LPS-IgG1	-24.96	13.63	0.067	0.0023	115.13
LPS-IgA	**-36.77**	**17.33**	**0.034[2]**	**0.0031**	**132.46**
LPS-IgM	-21.79	19.90	0.274	0.0008	68.23
PGN-IgG1	**-49.51**	**15.38**	**0.001[2]**	**0.0071**	**198.01**
PGN-IgA	-27.71	16.17	0.087	0.0020	105.61
PGN-IgM	-21.12	19.41	0.277	0.0008	67.63
LTA-IgG1	-16.72	16.56	0.313	0.0007	63.02
LTA-IgA	**-49.60**	**20.42**	**0.015[2]**	**0.0040**	**149.05**
LTA-IgM	**-39.64**	**18.62**	**0.034[2]**	**0.0031**	**134.02**
SCS	**-61.65**	**18.67**	**0.001[2]**	**0.0074**	**201.52**

[1] Name of antibody/antigen combination. Where KLH = keyhole limpet hemocyanin, LPS=lipopolysaccharide, LTA = lipoteichoic acid, PGN = peptidoglycan, IgA = Ab isotype IgA, IgG1 is Ab isotype IgG1 and IgM is Ab isotype IgM.
[2]Significantly different (with $p<0.05$)

Table 3.3. Regression coefficients (Reg.coef) with standard errors (SE) of best combination of antibodies (Ab) with and without somatic cell score (SCS) to predict productive lifetime (PL) and prediction of difference in days between animals with 10% lowest and 10% highest Ab and SCS (pred_contrast).

Ab	Reg.coef.	SE	P-value	R-square	Pred_ contrast
PGN-IgG1	-37.89	14.54	0.009	0.01	224.44
SCS	-38.08	17.96	0.034		
Without SCS					
PGN-IgG1	-40.40	14.43	0.005	0.009	207.22
LTA-IgA	-32.45	19.27	0.092		

3.4 Discussion

The aim of this study was to investigate a possible relation between levels of Ab isotypes to various antigens, including those to microbe-related antigens (LPS, LTA, PGN) as well as an antigen that likely was never seen before by the animal, and thus may be regarded as a NAb, in milk and the productive lifetime (in days) of dairy cows. With respect to LPS, LTA and PGN, It has to be kept in mind, that we have no information on the infection status of the cows and therefore cannot discriminate specific from natural antibodies for these antigens. Different regression analyses, therefore, were performed on a large group of cows. Additionally, we studied the effect of adding Ab information to somatic cell score (SCS), the current predictor for PL, and the genetic correlations between antibody levels and PL.

3.4.1 Antibodies vs Productive Lifetime

In our study, productive lifetime (PL) was measured as days between day of first calving and day of culling. Productive lifetime shows high variation since cows are culled for different reasons: fertility problems, feet and leg problems, but also due to farmers management choices (Beaudeau et al., 2000). Especially, the decisions of individual farmers are not easy to correct for. Differences in PL, therefore, are a reflection of a combination of different factors. Regardless of the difficulty to filter out the variation of farmer's management we were still able to find significant relationships between Ab levels, depending on the isotype and antigen-specificity, and productive lifetime.

Detailed analyses on the concept of using immune traits as markers for animal health characteristics are limited by the lack of large-scale recording of parameters from individual animals. It seems reasonable to assume that longevity is positively associated with a higher immune competence (equals better health status) of an animal (Neerhof et al., 2000, Samoré et al., 2003). It has to be kept in mind, however, that levels of an immune trait can be differentially related with a health trait. Levels of natural (auto-)antibodies binding nuclear antigens were positively correlated with life span, but negatively correlated with reproduction features in sheep (Graham et al., 2010). We showed that lower levels of Ab levels, including natural Ab (binding KLH) in milk, measured during first lactation, are associated with a longer productive lifetime. Given this association, our results seem in contrast with earlier studies on chickens (Star et al., 2007, Sun et al., 2011), which reported a positive relation between NAb levels in blood and the length of survival period of laying hens. In dairy cows, evidence was found that a higher level of SpAb, measured in blood, is associated with healthier animals or with animals that have a lower risk of developing a disease. Thompson-Crispi et al. (2013a and 2013b) found that higher levels of SpAb and NAb are associated with a decrease of mastitis incidence. Banos et al. (2013) suggested that higher levels of NAb measured in blood are associated with lower incidence of mastitis. Additionally, Machado et al. (2014), demonstrated that there is an association between higher levels of NAb levels in blood around parturition and improved uterine health in dairy cows. Ploegaert (2010a) suggested that higher levels of IgG1 binding KLH in milk, are protective for clinical mastitis in healthy cows. Finally, low phenotypic correlations were found between NAb in blood and milk (de Klerk et al., 2015), suggesting that the location of NAb measured: blood or milk, affect the relationship of Ab levels with disease risk or health status. In addition, higher levels of NAb were associated with lower affinity of specific antibodies (SpAb) and the other way

around (Parmentier et al., 2008). Levels of NAb, therefore, might not reflect an animal's health potential directly, but may be related to the health status of a cow at the moment of sampling. An infection will likely enhance Ab levels and change isotypes at a particular moment. Our results indicate that animals with higher Ab levels in milk at a certain moment, will have a shorter productive lifetime. Whether this reflects an activated immune system or enhanced susceptibility for infections resulting in earlier culling, remains to be further studied. Correction for a dilution effect for the amount of milk produced did not influenced the level of Ab measured.

Previously, antibody-mediated immune- (AMIR) and cell-mediated immune responsiveness (CMIR) were used as an indicator trait of the adaptive immune response of dairy cows (Mallard et al., 2011, Thompson-Crispi and Mallard, 2012a). The AMIR based on specific antibody responsiveness is important for the defence against extracellular pathogens, mostly involves the IgG1 isotype, and is characterized by higher levels of B-lymphocytes, which are important in production of antibodies. Higher AMIR and CMIR were both associated with a lower incidence of mastitis, but were genetically correlated in a negative fashion (Hine et al., 2011, Thompson-Crispi et al., 2012b). The role of NAb in AMIR or CMIR is yet unknown. All the above mentioned Ab levels studied were, however, measured in blood, whereas in the present study SpAb and NAb were measured in milk only.

In the present study multiple antigens were studied that can or had been encountered by the cow (LPS, LTA, PGN), or which likely will not be encountered (KLH) by the cow. PGN and LTA originate from the cell wall of gram-positive bacteria, whereas LPS is derived from gram-negative bacteria. At present it cannot be distinguished whether a cow has encountered such bacteria before, which impedes a distinction between natural and specific Ab to these antigens. The fore mentioned antigens tested have been shown to have immuno-modulatory effects (Parmentier et al., 2004), consequently variation in exposure as well as concurrent and previous infections would likely influence the Ab levels binding to these antigens. KLH, on the other hand, is a naive antigen, derived from the Californian sea limpet *Megathura crenulata*. We are not aware of cross reactive epitopes between KLH and infectious agents commonly present in the environment or intestinal microbiota. Thus, Ab binding to KLH can be assumed to be real NAb. No significant relations were found between KLH binding NAb and PL, which may likely rest on the unexplained large variation of PL.

Different Ab isotypes were studied. The present data showed that the IgA isotype significantly affected PL in most cases (Table 3.2), suggesting that IgA is the most promising antibody isotype in milk as indicator for PL. The IgM isotype had in most

cases the lowest power to predict PL. The earlier published higher heritabilities of IgM compared to other isotypes (Wijga et al., 2013, de Klerk et al., 2015) suggested that IgM is the more genetic component of the humoral immune system. IgM B cells not only provide the source of the B cells producing other isotypes (IgG1 and IgA) after isotype switching, but also serve as first line of defence within the immune system. Levels of IgM, therefore, might be less subject to environmental influences as opposed to the IgG and IgA isotypes , thus IgM levels are likely more important when looking at the relation with PL at a genetic level.

3.4.2 Antibodies vs Somatic Cell Score

Based on the relationships between productive lifetime and Ab in milk presented in this study it is concluded that Ab levels including NAb in milk show a pattern comparable to the pattern of SCS. Both Ab in milk and SCS showed a negative relation with PL in days. A higher level of SCS is associated with a higher incidence of mastitis, and, therefore, with a shorter PL. The predictive value of SCS was higher than the predictive value of a single Ab, probably because SCS is highly related to mammary gland infections (Sharma et al., 2011), which is a main reason for culling. When Ab and SCS were combined, however, adding information of Ab increased the prediction of PL. This means that Ab and SCS may reflect comparable processes going on within a cow. On the other hand, Ab seems to give additional information besides the information obtained from SCS. When SCS was left out of the stepwise regression analyses, Ab still had predictive power in the same order of magnitude compared to when SCS was included. Ab levels measured in milk may reflect immune processes and disorders within the cow, whereas SCS is a local mastitis related parameter, The present results indicate that Ab levels (in milk) can be a useful predictor for PL in addition to SCS, and can be used to increase accuracy of breeding values in breeding indices focussing on prediction of longevity.

3.5 Acknowledgements

Data used within present study is part of Dutch Milk Genomics Initiative. Authors would like to thank Tosca Ploegaert for providing data. Additionally, thanks to all the herd owners for their cooperation.

3.6 References

Banos, G., E. Wall, M. P. Coffey, A. Bagnall, S. Gillespie, G. C. Russell, and T. N. McNeilly. 2013. Identification of immune traits correlated with dairy cow health, reproduction and productivity. PloS one 8(6):e65766.

Baumgarth, N., Tung, J.W., Herzenberg, L.A. 2005. Inherent specifities in natural antibodies: a key to immune defence against pathogen invasion. Springer Semin Immunopathol. 26: 347-362.

Baumgarth, N. 2011. The double life of a B-1 cell: self-reactivity selects for protective effector functions. Nat. Rev. Immunol. 11:34-46.

Beaudeau, F., H. Seegers, V. Ducrocq, C. Fourinchon, and N. Bareille. 2000. Effect of health disorders on culling in dairy cows: a review and critical discussion. Proc. Annales de Zootechnie. EDP Science: 293-311.

De Klerk, B., B. J. Ducro, H. C. M. Heuven, I. Den Uyl, J. A. M. Van Arendonk, J. J. Van der Poel. 2015. Phenotypic and genetic relationships of bovine natural antibodies binding keyhole limpet hemocyanin in plasma and milk. J. Dairy Sci. 98:1-7.

Dunkelberger, J. R., and W. -C. Song. 2010. Complement and its role in innate and adaptive immune responses. Cell Res. 20(1):34-50.

Graham, A. L., A. D. Hayward, K. A. Watt, J. G. Pilkington, J. M. Pemberton, and D. H. Nussey. 2010. Fitness correlates of heritable variation in antibody responsiveness in a wild mammal. Science 330:662-665.

Hine, B., S. Cartwright, and B. Mallard. 2011. Effect of age and pregnancy status on adaptive immune responses of Canadian Holstein replacement heifers. J. Dairy Sci. 94:981-991.

Machado, V. S., M. L. S. Bicalho, R. O. Gilbert, and R. C. Bicalho. 2014. Short communication: Relationship between natural antibodies and postpartum uterine health in dairy cows. J. Dairy Sci. 97:7674-7678.

Mallard, B. A., H. Atalla, S. Cartwright, B. Hine, B. Hussey, M. Paibomesai, K. Thompson-Crispi, and L. Wagter-Lesperance. 2011. Genetic and epigenetic regulation of the bovine immunesystem: Practical implications of the high immune response technology. Pages 53-63. Proc. National Mastitis Council 50th Annual Meeting. National mastitis Council Verona WI.

Neerhof, H. J., P. Madsen, V. P. Ducrocq, A. R. Vollema, J. Jensen, and I. R. Korsgaard. 2000. Relationships between mastitis and functional longevity in

Danish Black and White dairy cattle estimated using survival analysis. J. Dairy Sci. 83:1064-1071.

Parmentier, H. K., W. J. A. Van Den Kieboom, M. G. B. Nieuwland, G. De Vried Reilingh, B. N. Hangalapura, H. F. Savelkoul, and A. Lammers. 2004. Differential effects of lipopolysacharide and lipoteichoic acid on the primary antibody response to keyhole limpet hemocyanin in chickens selected for high or low antibody responses to sheep red blood cells. Poult Sci. 83: 1133-1139.

Parmentier, H. K., G. De Vries Reilingh, and A. Lammers. 2008. Decreased specific antibody responses to α-gal-conjugated antigen in animals with pre-existing high levels of natural antibodies binding α-gal-residues. Poultry Sci. 78:918-926.

Ploegaert, T.C.W., B. J. Ducro, T. Verhoeven, J. J. Van der Poel, T. J. G. M. Lam, H. K. Parmentier, J. A. M. Van Arendonk, H. F. J. Savelkoul, and E. Tijlhaar. 2010a. Relation of natural antibodies with risk for mastitis and high somatic cell count in Dutch Holstein Friesian cows. In: Parameters for natural resistance in bovine milk (Phd Thesis), p 82-92. Wageningen University, Wageningen, The Netherlands. ISBN: 978-908585-827-0.

Ploegaert, T.C.W., B. J. Ducro, L. H. Oosterik, J. J. Van der Poel, T. J. G. M. Lam, H. K. Parmentier, J. A. M. Van Arendonk, H. F. J. Savelkoul, and E. Tijlhaar. 2010b. Relation of natural antibodies in milk of Holstein Friesian heifers with the risk for high somatic cell count and mastitis. In: Parameters for natural resistance in bovine milk (Phd Thesis), p 67-80. Wageningen University, Wageningen, The Netherlands. ISBN: 978-908585-827-0.

Ploegaert, T.C.W., Tijhaar, E., Lam, T.J.G.M., Taverne-Thiele, A., van der Poel, J.J., van Arendonk, J.A.M., Savelkoul, H.F.J, Parmentier, H.K. 2011. Natural antibodies in bovine milk and plasma: Variability among cows, repeatability within cows, and relation between milk and plasma titers. Vet. Immunol. Immunopathol. 144, 88-94.

Rogers, G. W., J. A. M. van Arendonk, B. T. McDaniel. 1988. Influence of involuntary culling on optimum culling rates and annualized net revenue. J. Dairy Sci. 71:3463-3469.

Samoré, A. B., M. D. P. Schneider, F. Canavesi, A. Bagnate, and F. A. Groen. 2003. Relationship between somatic cell count and functional longevity assessed using survival analysis in Italian Holstein-Friesian cows. Livest Prod Sci. 80 (3):211-220.

Sewalem, A., F. Miglior, and B. J. Van Doormaal. 2006. Analysis of the relationship between somatic cell score and functional longevity in Canadian dairy cattle. J. Dairy Sci. 89(9):3609-3614.

Sharma, N., N. Singh, and M. Bhadwal. 2011. Relationship of somatic cell count and mastitis: an overview. Asian-Aust. J. Anim. Sci. 24:429-438.

Star, L., Frankena, K., Kemp, B., Nieuwland, M.G.B., Parmentier, H.K. 2007. Natural humoral immune competence and survival in layers. Poultry Sci. 86: 1090-1099.

Sun, Y., H .K. Parmentier, K. Frankena, J .J. van der Poel. Natural antibody isotypes as predictors of survival in laying hens. Poultry Science 90: 2263-2274, 2011.

Thompson-Crispi, K.A., B. A. Mallard. 2012a. Type 1 and Type 2 immune response profiles of commercial dairy cows in 4 regions across Canada. Can. J. Vet. Res. 76(2):120-128.

Thompson-Crispi, K. A., A. Sewalem, F. Miglior, and B. A. Mallard. 2012b. Genetic parameters of adaptive immune response traits in Canadian Holsteins. J. of Dairy Sci. 95: 401-409.

Thompson-Crispi, K. A., F. Miglior, B. A. Mallard, 2013a. Incidence rates of clinical mastitis among Canadian Holsteins classified as high, average, or low immune responders. Clinical and Vaccine Immunol. 20:106-112.

Thompson-Crispi, K.A., F. Miglior, B. A. Mallard, 2013b. Genetic parameters for natural antibodies and associations with specific antibody and mastitis in Canadian Holsteins. J. Dairy Sci. 96: 3965-3972.

Van Knegsel, A. T. M., M. Hostens, G. de Vries Reilingh, A. Lammers, B. Kemp, G. Opsomer, and H. K. Parmentier. 2012. Natural antibodies related to metabolic and mammary health in dairy cows. Prev. Vet. Med. 103:287-297.

Van Pelt, M.L., T.H.E. Meuwissen, G. de Jong and R.F. Veerkamp. 2015. Genetic analysis of longevity in Dutch Dairy cattle using random regression. J. Dairy Sci. 98:4117-4130.

Vollema, A.R. 1998. Longevity of dairy cows: a review of genetic variances and covariances with conformation. Animal Breeding Abstracts 66:782-802.

Wijga, S., Bovenhuis, H., Bastiaansen, J.W.M., van Arendonk, J.A.M., Ploegaert, T.C.W., Tijhaar, E., van Poel, J.J. 2013. Genetic parameters for natural antibody isotype titers in milk of Dutch Holstein-Friesians. Anim. Genet. 44: 485-492.

4

Relation between antibodies measured in milk and estimated breeding values for longevity of Dutch dairy cows

B. de Klerk[*], B.J. Ducro[*], E. Mullaart[†], E. Koenen[†], J.A.M. van Arendonk[*], H.K. Parmentier[‡], J.J. van der Poel[*]

[*]Animal Breeding and Genomics Centre, Wageningen University, Wageningen, The Netherlands, P.O. Box 338
[†] CRV BV, Arnhem, The Netherlands
[‡]Adaptation and Physiology Group, Wageningen University, Wageningen, The Netherlands, P.O. Box 338

Submitted to Journal of Animal Breeding and Genetics

Abstract

Longevity of dairy cows is often used in breeding programs as selection criteria. Longevity is described as productive lifetime (PL), the time between first calving and actual culling. Since (real) PL can only be measured after culling, traits that can be measured during life and predict PL, are used. Thus, to improve the accuracy of estimated breeding values (EBV's) for PL, health parameters like somatic cell count, or claw health are used as predictors for PL. Antibodies (Ab), which are a major component of the immune system, might be useful to include as predictor for PL, but little is known of relationships between Ab and PL based on breeding values. The current study addresses the relation between direct and indirect EBV for PL and Ab levels measured in milk of first parity dairy cows. Levels of three Ab isotypes (IgG1, IgA and IgM) in 1456 heifers, were measured, binding four antigens: lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN), respectively, and presumably natural antibodies (NAb) binding keyhole limpet hemocyanin (KLH). Overall, cows with lower Ab levels in milk, have a higher EBV for both indirect- and direct longevity. These results imply that Ab levels are related with EBV's of PL and Ab levels in milk might therefore be useful as early predictors for longevity.

Keywords: antibody, longevity, breeding value, dairy cow

4.1 Introduction

Longevity is an important trait in dairy cattle breeding. It is still a challenge, however, to accurately estimate breeding values for longevity of an animal which is still alive, since longevity can only be obtained after an animal is culled. Additionally, longevity depends on many different factors with strong variation like farm management, disease resistance, environment, etc. Antibodies (Ab), measured in dairy cows, have been found to be related to different important health traits, like mastitis (Ploegaert et al. 2010b, Thompson-Crispi et al. 2013). Antibody levels, therefore, might be a relevant tool to improve the accuracy to predict longevity.

Antibodies play an important role within the immune system, and can be divided into specific antibodies (SpAb) and natural antibodies (NAb). Specific antibodies are part of the specific adaptive immune system and are produced by B2-cells in response to antigenic challenge. Natural antibodies are produced by B1-cells and are regarded as part of innate immunity, they are more responsible for the first line defense and are thought to be an important factor of natural resistance. Both SpAb and NAb occur in different isotypes like IgA, IgG and IgM, which are all found to be heritable traits (Wijga et al. 2013). Natural antibodies bind naïve antigens which an animal has never seen before, therefore, we assume Ab binding to *Megathura crenulata*-derived keyhole limpet hemocyanin (KLH), to be actual NAb. The heritabilities of both NAb and SpAb are comparable. Heritabilities for SpAb isotypes binding different antigens, range from 0.08 – 0.49 (Wijga et al. 2013, Thompson-Crispi et al. 2013). Heritabilities for different NAb isotypes binding KLH vary between 0.09-0.40 (Wijga et al. 2013, De Klerk et al. 2015, Thompson-Crispi et al. 2013). The level of the heritabilities for both SpAb and NAb, indicate that Ab can be a promising tool within breeding strategies.

Longevity, on the other hand, has a low heritability (0.01-0.03) (Vollema 1998 and van Pelt et al. 2015), due to large environmental variation, mainly caused by farm management, feeding regimes and housing differences. Hence, estimated breeding values for longevity, that are often used for breeding strategies, are not always as accurate as desired. The accuracy of these breeding values, however, can be improved by adding more reliable predictors for longevity.

A novel reliable predictor of longevity might be the levels of antibody (Ab) in blood or milk. So far only relations between phenotypic longevity (in days) and phenotypic values for Ab levels in milk have been estimated (De Klerk et al. submitted). however, the relation between antibody level and the genetic potential of an animal's longevity has not been investigated. The aim of this study, therefore,

is to (1) describe the relation between Ab levels measured in milk (SpAb and NAb) and EBV's for indirect longevity of first lactation cows, and (2) describe the relation between Ab levels measured in milk (SpAb and NAb) and EBV's for direct longevity.

4.2 Material and Methods

4.2.1 Animals

Information on all cows was collected as part of the Dutch Milk Genomics Initiative. For this study, 1456 first parity cows, originated from 339 Dutch dairy herds, were analysed. The number of cows from each herd varied from one to ten. The antibody levels were measured in milk samples as described by Ploegaert (2010b). Milk samples were collected between Feb 9th and May 3rd 2005. All cows first calved between the age of 54.6 and 90.7 months, with an average of 64.8 months. Most cows (61.6%) calved in autumn (September-November 2004), 36 % of the cows calved in summer (June-August 2004) and just 2.4% of the cows calved in winter (December 2004 – February, 2005). Days in lactation varied between 63 days and 267 days with a mean of 167 days. The cattle cooperative CRV (Arnhem, The Netherlands) provided information on culling dates of cows and somatic cell count (SCC) of milk samples. Milk records used were chosen based on closest date to sampling date. All 1456 cows were culled between the 1st of April 2005 and the 20th of December 2012. Longevity was calculated as productive lifetime (PL); i.e. the difference in days between the day of first calving and the day of culling. Somatic cell score (SCS) from milk samples was calculated as the natural logarithm of SCC/1000.

4.2.2 Antibodies

Levels of antibodies (Ab) were measured in morning milk samples by indirect ELISA, as described by Ploegaert et al. 2010 and De Klerk et al. (submitted). In short: plates were coated with *Megathura crenulata*-derived keyhole limpet hemocyanin (KLH, MP Biomedicals, Solon, OH), , *Eschericia coli*-derived lipopolysaccharide, (LPS, L2880,serotype O55:B5, Sigma-Aldrich Inc. St. Louis, MO), *Staphylococcus. aureus*-derived lipoteichoic acid (LTA, L2515, Sigma-Aeldrich), *Staphylococcus aureus*-derived peptidoglycan (PGN, Bio-Chemika, Buchs, Switzerland). Binding of Ab to LTA, LPS, PGN, and KLH, respectively, was detected using 1:16,000 diluted sheep anti-bovine IgG1 (Serotec, Düsseldorf, Germany), 1:16,000 diluted sheep anti-bovine IgM (Serotec), or 1:8,000 diluted sheep anti-bovine IgA (Serotec), all

coupled to horseradish peroxidase. Extinctions were measured with a Multiskan spectrophotometer (Flow, Irvine, UK) at a wavelength of 450 nm. Ab levels were calculated as titers, where titers were expressed as log2 values of the dilutions that gave an extinction closest to 50% of E*max* (highest mean extinction of a standard positive milk sample present in duplicate on every microtiter plate). A total of 98 different plates with 10 columns were randomly selected and used in the process of scaling the Ab titers. As a result, twelve Ab isotype titers binding four different antigens were obtained: Ab isotype IgM binding KLH (IgM-KLH); LPS (IgM-LPS), PGN (IgM-PGN), or LTA (IgM-LTA), Ab isotype IgG1 binding KLH (IgG1-KLH); LPS (IgG1-LPS); PGN (IgG1-PGN); LTA (IgG1-LTA); and Ab isotype IgA binding KLH (IgA-KLH); LPS (IgA-LPS); PGN (IgA-PGN); LTA (IgA-LTA).

4.2.3 Longevity

Estimated breeding values (EBVs) for longevity were provided by CRV (Arnhem, The Netherlands) and were obtained from the routine genetic evaluation of 2014. Two types of EBVs were used in this study. The first one (EBV_DL) concerned recordings of longevity only and the genetic evaluation was based on survival methods to account for censoring in the data (Vollema et al., 2000; Van der Linde et al., 2004). The second type (EBV_IL) is basically an index of breeding values of longevity and its so-called early predictors, udder depth, locomotion score and (log) somatic cell count. Weighing into the index was based on the genetic correlations with longevity which was, 0.22 for udder depth, 0.24 for locomotion score and 0.44 for (log) somatic cell count. The second type of breeding value is the breeding value routinely published by CRV and expressed in days.

4.2.4 Statistical analyses

Antibody levels were pre-adjusted using the following model:

$$Y_{ijkl} = \mu + b_1 \text{ x } DIM_i + b_2 \text{ x } AFC_j + BATCH_k + FARM_l + e_{ijkl}$$

Where the response variable Y represented the different Ab binding different antigens (i.e. IgM-KLH, IgM-LPS, IgM-LTA, IgM-PGN, IgG1-KLH, IgG1-LPS, IgG1-LTA, IgG1-PGN, IgA-KLH, IgA-LPS, IgA-LTA, IgA-PGN), μ was the population mean, DIM was covariate describing the effect of days in milk, with regression coefficient b_1, AFC was covariate describing the effect of age at first calving in days, with regression coefficient b_2, BATCH was fixed effect of day of analysis (where *k* was

batch-class with 1=day 1, 2=day 2), FARM was random herd effect and e was the random residual.
For Ab binding to antigen LTA same fixed effects were used, except for 'BATCH', since LTA Ab were tested on 9 different days instead of only 2. Therefore, in this case BATCH was fixed effect of day of analysis (where *k* was batch-class with 1=day 1 and 2=day 2, 3=day 3, 4= day 4, 5=day 5, 6=day 6, 7=day 7, 8=day 8 and 9=day 9).

4.3 Results

Antibodies used for this study had 3 different isotypes (IgA, IgG and IgM), and were tested against 4 different antigens (KLH, LPS, LTA and PGN). Two different breeding values (EBV's) were considered in this study. EBV_IL, which also included early-predictors for longevity, varied from -415 to +429 days with a mean of 58.89 and a standard deviation of 132.00 days. The second EBV used was EBV for direct longevity (EBV_DL) which varied from -387 to +389 days with a mean of 66.71 days and a standard deviation of 125.71 days. For both EBV_IL and EBV_DL a negative relation was found with Ab levels. Where animals with higher Ab levels measured during first lactation are predicted to have lower EBVs for PL. EBV_IL shows a significant negative relation with multiple Ab- and NAb-antigen combinations; KLH-IgA, KLH-IgM, PGN-IgA, PGN-IgM, LTA-IgA and LTA-IgG1 (Table 4.1). Based on the relation between Ab and EBV_IL: when Ab level rises with 1 point, a cow is expected to have a 3.25 and 12.39 days lower EBV_IL. When SCS rises with 1 point, predicted BV_IL of this cow will decrease with 22.71 days. The difference in EBV_IL between the 10% highest- and 10% lowest cows for Ab, varies between 7.45 and 36.14 days. The difference in EBV-IL between the 10% highest- and 10% lowest cows for SCS, was 74.23. EBV_DL shows a significant negative relation with only one NAb-antigen combination; KLH-IgM (Table 4.2). When Ab level rises with 1 point, the EBV_DL decreases between 0.84 and 9.29 days. When SCS lowers 1 point, the EBV-DL will decrease with 4.53 days. Difference in EBV-DL between the 10% highest- and the 10% lowest cows for Ab, ranges between 3.37 and 27.09 days. For SCS, the cows with 10% highest- and 10% lowest values for SCS had a decrease of EBV_DL of 14.80 days. For both EBV-IL and EBV_DL, KLH-IgA showed a slightly different pattern; a higher level of KLH-IgA is associated with a higher EBV for longevity.

Table 4.1. Regression coefficients between Ab-antigen combination and SCS, and indirect EBV (EBV_IL) with standard errors (s.e.), p-values and prediction of difference in EBV_IL between 10% highest en 10% lowest animals for Abs and SCS (pred_contrast).

Ab[1]	Regression coefficient	s.e.	p-value	Pred_ contrast
KLH-IgG1	1.96	3.17	0.537	7.45
KLH-IgA	**-10.53**	**3.65**	**0.004[2]**	**34.96**
KLH-IgM	**-12.39**	**4.16**	**0.003[2]**	**36.14**
LPS-IgG1	-3.25	2.67	0.224	14.99
LPS-IgA	-5.93	3.39	0.081	21.37
LPS-IgM	-4.71	3.90	0.228	14.75
PGN-IgG1	-4.70	3.01	0.118	18.80
PGN-IgA	**-8.60**	**3.17**	**0.007[2]**	**32.77**
PGN-IgM	**-7.55**	**3.80**	**0.047[2]**	**24.16**
LTA-IgG1	**-7.31**	**3.24**	**0.024[2]**	**27.53**
LTA-IgA	**-10.67**	**4.00**	**0.008[2]**	**32.06**
LTA-IgM	-3.66	3.65	0.317	12.38
SCS	**-22.71**	**3.62**	**<0.0001[2]**	**74.23**

[1] Name of antibody/antigen combination. Where KLH = keyhole limpet hemocyanin, LPS=lipopolysaccharide, LTA = lipoteichoic acid, PGN = peptidoglycan, IgA = Ab isotype IgA, IgG1 is Ab isotype IgG1 and IgM is Ab isotype IgM.
[2]Significantly different (with $p<0.05$)

4.4 Discussion

Within the dairy sector, breeders often use estimated breeding values (EBV's) for longevity to select their cows and bulls to breed for longer living cows. Estimated breeding values for longevity are not fully accurate, since longevity contains a large part of undefined environmental variance and therefore, is a low heritable trait. To improve the quality of longevity EBV's there is a need for more accurate predictors

of longevity. In this study three different Ab isotypes (IgA, IgG and IgM), binding four different antigens (KLH, LPS, LTA and PGN), were studied as predictor for

Table 4.2. Regression coefficients between Ab-antigen combination and direct EBV (EBV_DL) with standard errors, p-values and prediction of difference in EBV between 10% highest en 10% lowest animals for Ab and SCS (pred_contrast).

Ab[1]	Regression coefficient	s.e.	p-value	Pred_contrast
KLH-IgG1	2.33	3.00	0.438	8.88
KLH-IgA	-5.96	3.45	0.085	19.78
KLH-IgM	**-9.29**	**3.93**	**0.018[2]**	**27.09**
LPS-IgG1	-3.14	2.25	0.214	14.49
LPS-IgA	-1.86	3.21	0.563	6.70
LPS-IgM	-2.64	3.69	0.473	8.29
PGN-IgG1	-0.84	2.84	0.767	3.37
PGN-IgA	-2.98	3.00	0.321	11.34
PGN-IgM	-4.62	3.59	0.199	14.79
LTA-IgG1	-3.57	3.06	0.244	13.45
LTA-IgA	-3.36	3.79	0.375	10.11
LTA-IgM	-1.35	3.45	0.697	4.55
SCS	-4.53	3.47	0.192	14.80

[1] Name of antibody/antigen combination. Where KLH = keyhole limpet hemocyanin, LPS=lipopolysaccharide, LTA = lipoteichoic acid, PGN = peptidoglycan, IgA = Ab isotype IgA, IgG1 is Ab isotype IgG1 and IgM is Ab isotype IgM.
[2]Significantly different (with $p<0.05$)

longevity. Levels of Ab were measured in milk samples of heifers, and compared to breeding values for longevity. Based on these expected performance parameters, we found a negative relation between the Ab levels and EBV's for longevity (both indirect and direct longevity). This suggests that animals with lower Ab levels in

milk, measured during first lactation, will be expected to live longer productive lives.

4.4.1 Antibodies

In this study antibody levels were measured in milk, since it is possible to only measure levels in milk, and still obtain information on genetic predisposition (De Klerk et al., 2015). High genetic correlations were found between NAbs measured in blood and milk. NAb or Ab levels measured in milk are cheaper to obtain and less labor intensive, compared to Ab levels obtained from blood, since it can be measured within the regular test procedure of milking cows. Measurement of Ab levels, therefore, can be an easy large scale and cheap tool to obtain more information for the prediction of a cow's productive lifetime.
The Ab used in this study were tested to bind to four different antigens (KLH, LPS, LTA and PGN). Antigens LPS, LTA and PGN are derived from bacterial membranes, which are likely present within the environment. A cow, therefore, might have encountered such antigens earlier in life. Levels of Ab to these antigens may then rest on cross-reactivity or specific seroconversion after challenge. Therefore, we cannot exclude that Ab binding LPS, LTA and PGN are specific antibody (SpAb).
The antigen KLH, on the other hand, is derived from the deep sea snail (*Megathura crenulata*), and therefore the most naïve antigen used within this study. Antibodies binding KLH likely are NAb. In the current study, levels of NAb binding KLH gave the strongest relations with EBV's for longevity (Table 4.1 and Table 4.2). This suggests that natural resistance of a cow is important in respect to its expected longevity and can be measured during early life (first lactation) using NAb. When more detailed survival analyses will be applied, and a heifer has a certain NAb level in milk binding to KLH, it can be predicted how long the cow most likely will continue her productive life. Based on this knowledge a farmer can make decisions on which cow to keep, or which cow to remove from the herd.

4.4.2 Antibodies vs SCC

In this study a negative relation was found between Ab levels and EBV's for longevity. The same trend was observed when comparing EBV's for longevity (direct and indirect) to somatic cell count (SCC) (Tables 4.1 and 4.2). It was expected that SCC has a negative relation with longevity; a high SCC is strongly correlated with incidence of mastitis (Samoré et al., 2003) a prominent reason for removal of cows. For Ab it is still not fully known if lower or higher levels, either in

blood or milk, and what isotypes directed to certain antigens are most useful when looking at longevity. However, this and a previous study (De Klerk et al submitted), indicated that lower Ab levels in milk of all three isotypes are more preferred when selecting cows for a longer productive life length. NAb bind to naïve antigens, but are also involved in activating the specific immune system for further reactions and formation of memory cells linking innate and adaptive immunity (Ochsenbein and Zinkernagel, 2000). When the level of NAb is high, lower SpAb responses to new antigens may be found (Sinyakov et al., 2002) whereas low levels of NAb in calf plasma were accompanied by higher SpAb responses (Mayasari et al., 2015). It remains to be studied whether lower (N)Ab levels (in milk) reflect lower immune competence, whereas high NAb levels may reflect inability to cope with infection or damage. This may also apply for SCC; a too low number of somatic cells present in the udder may not be not preferable (Suriyasathaporn et al., 2000).

4.4.3 EBV's for longevity vs Ab levels

Generally, EBV's for longevity are estimated based on different predictors for longevity, such as fertility, mastitis, claw-problems, milk production and many other traits (Pfeiffer et al. 2015, De Jong et al. 1999, Essl, 1998). In this study two different EBV's for longevity were used to compare to Ab levels. First, the indirect EBV for longevity was estimated (EBV_IL) based on predictors as mentioned above. Second, the direct EBV for longevity was estimated (EBV-DL), which was based on direct longevity without other predictors. From the analyses based on the EBV_IL some Ab showed a significant relationship; a lower Ab level or NAb level refers to a higher EBV for longevity. This means that cows which have low antibody levels, are expected to live a longer productive life. The same trend was seen when Ab levels were compared to the direct EBV's for longevity. In case of EBV_DL, however, only levels of natural antibody (Ab binding KLH) showed a significant relation. As mentioned before, we hypothesize that NAb binding KLH refer to the first line of defense of an animal's immune system, and might therefore be related to the natural resistance of a cow. This confirms the theory that, when not correcting for environmental factors, relevant at moment of culling (what is the case when using estimated breeding values for indirect longevity) the most stable relation can be found between natural antibody isotype IgM. In this study, especially KLH-IgA and KLH-IgM showed a significant relation with EBV for direct longevity. Earlier (de Klerk et al submitted), NAb binding KLH did not show a significant relation with longevity, when analyzed at the phenotypical level. It is possible, however, based on the current antigens and antibodies tested, that EBV's, reflect the genetic

potential of individual cows for maintaining PL. Since both Ab- and NAb levels are heritable (De Klerk et al. 2015, Wijga et al. 2013, Ploegaert et al. 2010b), and are assumed to be related with different health traits in dairy cows (Denholm et al. 2015, Machado et al. 2014, Thompson-Crispi et al 2012, Van Knegsel et al. 2012), it is likely that they can give information about the potential to stay healthy. The IgM isotype was found to be the most heritable among the different Ab (De Klerk et al. 2015, Wijga et al. 2013) Therefore, based on this study we suggest that IgM levels binding different antigens add to identification of cows with the genetic potential to have a longer PL. Based on this information, farmers can make decisions on removal of cows early in their productive life.

We conclude that there is a negative relation between EBV's for longevity (both indirect and direct) and Ab levels measured in milk of heifers. When an animal has lower Ab levels during early life (first lactation), it has a higher EBV for longevity, and is therefore assumed to live a longer (productive) life. These results imply that antibodies can be useful as early predictors for longevity, and might be a suitable candidate to be used as additional predictor for longevity when estimating EBV for longevity.

4.5 References

De Jong, G., A. R. Vollema, S. van der Beek, and A. Harbers. 1999. Breeding value for functional longevity in the Netherlands. Page 68 in Proc. Int. Workshop on Genetic Improvement of Functional Traits in cattle, Longevity, Jouy-en-Josas, France. INTERBULL Bull. No. 21. Int. Bull Eval. Serv., Uppsala, Sweden.

De Klerk, B., B. J. Ducro, H. C. M. Heuven, I. Den Uijl, J. A. M. Van Arendonk, J. J. Van der Poel. 2015. Phenotypic and genetic relationships of bovine natural antibodies binding keyhole limpet hemocyanin in plasma and milk. J. Dairy Sci. 98:1-7.

Denholm, S. J., T. N. McNeilly, G. Banos, M. P. Coffey, G. C Russell, A. Bagnall, M. C. Mitchell and E. Wall. 2015. Relationship between immune traits found in the blood and milk of Holstein Friesian dairy cows. Proceedings of 66th Annual meeting of European Association of Animal Production, 31 August- 4 September 2015, Warsaw, Poland.

Essl, A., 1998. Longevity in dairy cattle breeding: a review. Livestock Prod. Sci. 57: 79-89.

Machado, V. S., M. L. S., Bicalho, R. O. Gilbert and R. C. Bicalho. 2014. Short communication: Relationship between natural antibodies and postpartum uterine health in dairy cows. J. Dairy Sci. 97:7674-7678.

Van der Linde, C, G. de Jong en A. Harbers. 2004. Using a piecewise Weibull mixed model in the genetic evaluation for longevity. Interbull Bulletin 2004, 32:157-62

Mayasari N., G. de Vries Reilingh, M. G. B. Nieuwland, G. J. Remmelink, H. K. Parmentier, B. Kemp, A. T. M. van Knegsel. 2015. Effect of maternal dry period length on colostrum immunoglobulin content, natural and specific antibodies titers and development of calves. J. Dairy Sci. 98: 3969-3979

Ochsenbein, A. F. and R. M. Zinkernagel. 2000. Natural antibodies and complement link innate and acquired immunity. Immunol. Today 21:624-630.

Pfeiffer, C., C. Fuerst, V. Ducrocq and B. Fuerst-Walt. 2015. Short communication: Genetic relationships between functional longevity and direct health traits in Austrian Fleckvieh cattle. J. Dairy Sci. 98:7380-7383.

Ploegaert, T. C. W., B. J. Ducro, L. H. Oosterik, J. J. Van der Poel, T. J. G. M. Lam, H. K. Parmentier, J. A. M. Van Arendonk, H. F. J. Savelkoul, and E. Tijhaar. 2010. Relation of natural antibodies in milk of Holstein Friesian heifers with the risk for high somatic cell count and mastitis. In: Parameters for natural resistance in bovine milk (Phd Thesis), p 67-80. Wageningen University, Wageningen, The Netherlands. ISBN: 978-908585-827-0.

Ploegaert, T. C. W., S. Wijga, E. Tijhaar, J. J. van der Poel, T. J. G. M. Lam, H. F. J. Savelkoul, H. K. Parmentier, J. A. M. van Arendonk. 2010b. Genetic variation of natural antibodies of Dutch Holstein-Friesian cows. J. Dairy Sci. 93:5467-5473.

Samoré, A. B., M. D. P. Schneider, F. Canavesi, A. Bagnate, and A. F. Groen. 2003. Relationship between somatic cell count and functional longevity assessed using survival analysis in Italian Holstein-Friesian cows. Livest Prod Sci. 80 (3):211-220.

Sinyakov, M. S., M. Dror, H. M. Zhevelev, S. Margel, and R. R. Avtalion. 2002. Natural antibodies and their significance in active immunization and protection against a defined pathogen in fish. Vaccine 20(31):3668-3674.

Suriyasathaporn, W., Y. H. Schukken, M. Nielen, and A. Brand. 2000. Low somatic cell count: a risk factor for subsequent clinical mastitis in a dairy herd. J. Dairy Sci. 83(6):1248-1255.

Sun, Y., H. K. Parmentier, K. Frankena, J. J. van der Poel. 2011. Natural antibody isotypes as predictors of survival in laying hens. Poultry Science 90: 2263-2274.

Thompson-Crispi, K. A., B. Hine, M. Quinton, F. Miglior and B.A. Mallard. 2012. Short communication: Association of disease incidence and adaptive immune response in Holstein dairy cows. J. Dairy Sci. 95:3888-3893.

Thompson-Crispi, K. A., F. Miglior, and B. A. Mallard. 2013. Genetic parameters for natural antibodies and associations with specific antibody and mastitis in Canadian Holsteins. J. Dairy Sci. 96: 3965-3972.

Van Knegsel, A. T. M., H. Hostens, G. de Vries Reiling, A. Lammers, B. Kemp, G. Opsomer and H. K. Parmentier. 2012. Natural antibodies related to metabolic and mammary health in dairy cows. Preventive Veterinary Medicin 103:287-297.

Van Pelt, M. L., T. H. E. Meuwissen, G. de Jong and R. F. Veerkamp. 2015. Genetic analysis of longevity in Dutch dairy cattle using random regression. J. Dairy Sci. 98:4117-4130.

Vollema, A. R., 1998. Longevity of dairy cows: a review of genetic variances and covariances with conformation. Animal Breeding Abstracts 66:782-802.

Vollema, A. R., 2001. Genetic evaluation for longevity of Dutch dairy bulls. J. Dairy Sci: 83: 2629–2639.

Wijga, S., H. Bovenhuis, J. W. M. Bastiaansen, J. A. M. van Arendonk, T.C.W. Ploegaert, E. Tijhaar, and J. J. van Poel. 2013. Genetic parameters for natural antibody isotype titers in milk of Dutch Holstein-Friesians. Anim. Genet. 44: 485-492.

5

A genome-wide association study for natural antibodies measured in blood of Canadian Holstein cows

B. de Klerk[*], K. A. Thompson Crispi[2], M. Sargolzaei[3,4], B.J. Ducro[1], J.A.M. van Arendonk[1], J.J. van der Poel[1], B.A. Mallard[3,5]

[1]Animal Breeding and Genomics Centre, Wageningen University, Wageningen, The Netherlands, P.O. Box 338

[2] Trouw Nutrition Agresearch, Guelph, Ontario, Canada

[3]Centre for Genetic Improvement of Livestock, University of Guelph, Guelph, Ontario Canada

[4]Semex Alliance, Guelph, Ontario, Canada

[5]Department of Pathobiology, Ontario Veterinary College, Genetic improvement of livestock, University of Guelph, Guelph N1G 2W1, Ontario, Canada

To be submitted

Abstract

Natural antibodies (NAb) are an important component of the innate immune system, and fight infections as a first line defence. NAb are poly-reactive and can respond non-specific to naive antigens. Therefore, natural antibodies may be a key trait when evaluating an animal's potential natural disease resistance. Variation in natural antibodies is caused by both genetic and environmental factors. In this study genetic parameters of NAb were estimated and a genome-wide association study (GWAS) was performed to gain further understanding on the genes that are responsible for the observed genetic variation in natural antibody levels. Blood samples of in total 727 cows from 7 farms were studied. Natural antibody levels binding keyhole limpet hemocyanin (KLH) were determined via indirect ELISA. Both immunoglobulin isotype IgG and IgM were tested. Cows were genotyped for 45.187 markers. Each individual marker was tested to detect genetic variation in natural antibody levels. Results show heritabilities of 0.13± 0.062 (IgG) and 0.27± 0.076 (IgM). Furthermore, only significant associations were found for immunoglobulin isotype IgG, and al significant associations were located on chromosome 21. Genomic regions that were identified in this study contained immune-response related genes like IgH, TRAF3 and TP53BP1. Therefore these regions are suggested to contain candidate gene(s) involved in natural antibody expression in dairy cows, both from the gene positional and gene functional perspective.

Keywords: genome-wide-association study, natural antibody, dairy cattle

5.1 Introduction

Over the last decades, breeding in dairy cattle mainly focused on production and fertility traits, with less emphasis on health traits. Health problems, however, can cause substantial economic losses to the dairy industry. The economic losses, together with the rising awareness of animal welfare, increased herd size, and less attention for individual animals, have led to an increased need to focus more on health traits. So far, health parameters used in dairy cattle breeding programs mainly considered some specific health parameters such as, milk quality related parameters, like somatic cell count or score (SCC or SCS) and clinical mastitis resistance, or claw related health parameters, or ketosis. Somatic cell score is generally used for breeding indices and has a heritability between 0.10 and 0.17, when based on lactation means (Pryce et al., 1998; Rupp and Boichard, 1999 and 2003; Carlen et al., 2004). SCS is associated with mastitis (Harmon et al., 1994) and is, therefore, related to udder-quality, rather than the total health status of a cow. Another potential shortcoming of SCS as a health indicator trait is that short-duration infections may be difficult to identify simply from increased average SCS during lactation, because SCS is often recorded at approximately monthly intervals.
Currently, no parameters in the breeding index select for the overall health status (immune capacity) of a cow, therefore, there is a demand for finding other parameters associated with overall natural resistance in dairy cows. Natural antibodies (NAb) might be a good candidate to use as parameter for natural resistance in dairy cows. NAb are part of the innate immune system, are produced by B-cells and can be found in animals without prior, intentional exposure to antigens (Baumgarth et al., 2005). In this way, NAb play an important role in the first line of defence against different kinds of infections (Casali and Schettino, 1996). They are polyreactive to bind different conserved structures like carbohydrate, nucleic acid and phospholipids (Boes, 2000). Different NAb isotypes exist, where isotype IgM is most commonly present, and isotypes IgG and IgA are mostly found to a lesser extent (Kohler et al 2003). Additionally, NAb provide a link between innate and adaptive immune system (Ochsenbein and Zinkernagel, 2000). In dairy cows, NAb are measurable in both blood- and milk samples (de Klerk et al., 2015; Ploegaert et al., 2011).
Many studies have shown that it is possible to select for healthier cows based on adaptive immune response traits (Wagter et al., 2000; Hernadez et al., 2003; Thompson-Crispi et al., 2012a; Thompson-Crispi et al., 2013b). In these studies, the relationships between economically important diseases (like mastitis, metritis, displaced abomasums (DA) and retained placenta) of dairy cows and their immune

response were studied and it was proven that specific antibodies (SpAb), which are part of the adaptive immune system, are useful biomarkers for disease resistance. Cows were tested for two different adaptive immune responses, antibody mediated (AMIR) and cell mediated immune response (CMIR) (Thompson-Crispi et al., 2013b). A decreased incidence of mastitis was found for cows with a high AMIR, CMIR and overall immune response. Moreover, a higher incidence of metritis was found for cows with a low CMIR and cows with low overall immune response had an increased incidence of retained foetal membrane and displaced abomasum (Thompson-Crispi et al., 2013b). As a conclusion, SpAb are a reliable parameter to include in breeding programs in order to improve disease resistance in dairy cows, since SpAb were found to be related to disease incidence. However, SpAb of the adaptive immune response are only measured after immunization of the cows, whereas NAb can be measured prior to immunization, and therefore may be favoured in a breeding program.

Recently, NAb have been studied as a predictor for different diseases in dairy cows. NAb levels binding KLH and LPS were positively related to negative energy balance (NEB), metabolic health and diet composition in early lactation (Van Knegsel et al. 2007). Van Knegsel et al. (2012) found a positive correlation between mastitis incidence and NAb binding to self-antigen myosin, and a negative correlation between mastitis incidence and NAb binding to self-antigen transferin. Banos et al. (2013) hypothesized that overall, higher NAb levels (when binding to KLH), were associated with improved capacity of the innate immune system to respond to pathogen challenges. However, they also found that a poorer nutritional status was related with higher NAb levels. Ploegeart et al. (2010) found that higher NAb levels (especially isotype IgG binding KLH) prevent the chance of developing mastitis later in lactation. Most results of these studies found that a higher level of NAb were associated with lower incidence of different diseases like mastitis, negative energy balance and ketosis.

Variation in NAb can result from both genetic and environmental factors. NAb are found to be heritable. Heritability estimates of NAb levels measured in blood or milk of dairy cows, ranges from 0.08 – 0.45 (Ploegaert et al., 2010b, Wijga et al., 2013, Thompson-Crispi et al., 2013 and De Klerk et al 2015). Where isotype IgM overall has highest heritability estimates (0.18 – 0.45) and isotype IgG has lower heritability estimates (0.08- 0.31). Additionally, NAb levels measured in blood serum had higher heritability estimates (0.15-0.25) compared to NAb levels measured in milk (0.08-0.23) (De Klerk et al., 2015). The relatively high heritability (especially for IgM isotype) shows potential for effective genetic selection. Due to the heritability of overall adaptive immune responsiveness (0.25-0.35) animals can

be genetically selected based on their immune response (Thompson-Crispi et al., 2012c). However, environmental factors (like herd, nutrition, housing) remain an important source of variation when it comes to animals health parameters.
The goal of present study was to identify regions of the genome important in the regulation of NAb levels of the IgM and IgG isotypes. Results are expected to provide a further step in unravelling the genetic control of NAb, which is, given the resemblances in NAb between species, not only relevant for dairy cattle but also for many other species. To our knowledge, no earlier genome-wide association studies (GWAS) on NAb, measured in single blood serum samples of the dairy cow, were published so far.

5.2 Material & Methods

5.2.1 Animals and Phenotypes

Blood serum samples were taken of 727 cows from 7 herds in Ontario, Canada. Cows were on average 131 days in lactation when the blood samples were taken, with a range from 0 to 423. Cows had on average a parity of 1.9, ranging from 0 to 12. The average number of cows per farm was 100, ranging from 60 to 160 cows per farm. The cows were sired by 243 different bulls and derived from 528 dams; varying from 1 to 26 offspring per sire and 1 to 11 offspring per dam. The pedigree contained 11,155 animals and was provided by the Canadian Dairy Network (CDN; Guelph, Ontario, Canada).
Immune response phenotypes used in this study were based on natural antibody response. Natural antibody of the isotype IgG and IgM were tested against the model antigen keyhole limpet hemocyanin (KLH). To obtain the natural antibody phenotypes an indirect ELISA procedure was used, as described by Thompsom-Crispi (2013a): Flat-bottomed 96 well polystrene plates were coated with 5 µg/ml Keyhole Limpet Hemocyanin (KLH) (MP Biomedicals, Solon, OH) in carbonatebicarbonate buffer (pH 9.6), and incubated over night at a temperature of 4°C. Next day plates were washed 3 times with PBS and 0.05% Tween 20 (Sigma-Aldrich Canada Ltd. , Oakville, ON, Canada) (wash buffer pH 7.4) and blocked with PBS, 3% Tween 20, 1,5% BSA and 1.5% FCS for 1 hour at room temperature (RT) then washed again 3 times. Four serial dilutions starting with 1/40 of the serum samples in wash buffer were added to the plate and incubated for 2 hours at RT. Plates were wash 5 times and the secondary antibodies conjugated to alkaline phosphatase dissolved in Tris-Tween buffer with 0.05% Tween 20 (pH 7.4) were

added to the plates: either 1: 10.000 monoclonal anti-bovine IgG from mouse ascites fluid (Sigma-Aldrich, St. Louis. MO, USA) or 1:5000 anti-bovine IgM produced in sheep (Bethyl Laboratories, Montgomery, TX, USA) and incubated for 1 hour at RT. All wash steps were performed with ELx405 Auto Plate washer (Biotek Instruments Inc., Winooski, USA). Substrate (p-nitrophenyl phosphate) (Sigma-Aldrich Canada Ltd, Oakville, On, Canada) was added and incubated for about 30-60 minutes. Optical density (OD) values at 405nm were obtained using EL808 plate reader (BioTek Instruments Inc., Winooski, VT, USA). Optical density values were corrected to the rolling mean of the positive controls for each plate to account for day and plate variation, as described by Heriazon et al. (2009). The dilutions of the corrected OD values were summed and duplicates averaged for statistical analysis. Natural antibody titers, both IgG and IgM isotype, were log-transformed to accomplish normality. After removing outliers for both IgG and IgM by Median Absolute Deviation method (considering three equivalent standard deviations), the final data set consisted of 681 cows with both genotypes and phenotype.

5.2.2 Genotyping and Quality Control

DNA was extracted from hair follicles and genotyping was performed with the Illumina Bovine SNP50 BeadChip by Zoetis Canada. The initial dataset contained 45,187 SNP markers that used in routine official genomic evaluation in Canada by the Canadian Dairy Network (Guelph, ON, Canada). Details of quality control were explained in Wiggans et al. (2009). In the present study SNPs located on the X chromosome were not included and due to relatively small sample size, SNPs with MAF < 1% in the 681 cows were excluded resulting in 43,283 SNPs for GWAS. The sporadic missing genotypes were imputed using 50,000 reference Holsteins from the CDN database by FImpute software (Sargolzaei et al. 2014).

5.2.3 Statistical analysis

The association of the individual natural antibody levels with each individual SNP was estimated following a univariate mixed linear model:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{w}\beta + \mathbf{Zg} + \mathbf{e}$$

where **y** is an observation vector, **b** is a vector of fixed effects including overall mean, days in milk (classes: 1= 0-20dim, 2= 21-105 dim, 3= 106-235dim , 4 > 235dim), parity (classes: 0= heifers before calving, 1= parity 1, 2= parity 2, 3= parity 3 and 4= parity 4 and higher), herd (classes: 1 to 7), β is the gene substitution effect for the SNP, **g** is the random genetic effects, **e** is the random residual effects, **X** and **Z** are incidence matrices relating elements of **b** and **g**, respectively, to **y**, and **w** is a vector of centred genotypes (i.e. c_i - 2p ; where p is the allele frequency of the SNP and c_i is the genotype of *ith* individual coded as 0=BB, 1=AB, 2=AA).

The co-variance matrix for the vector **y** is:

$$\mathbf{V} = \mathbf{G}\boldsymbol{\sigma}_{\boldsymbol{g}}^{\mathbf{2}} + \mathbf{I}\boldsymbol{\sigma}_{\boldsymbol{e}}^{\mathbf{2}}$$

With $\mathbf{g} \sim N(\mathbf{0}, \mathbf{G}\boldsymbol{\sigma}_{\boldsymbol{g}}^{\mathbf{2}})$ and $\mathbf{e} \sim N(\mathbf{0}, \mathbf{I}\boldsymbol{\sigma}_{\boldsymbol{e}}^{\mathbf{2}})$, where $\boldsymbol{\sigma}_{\boldsymbol{g}}^{\mathbf{2}}$ and $\boldsymbol{\sigma}_{\boldsymbol{e}}^{\mathbf{2}}$ denote variance of random genetic effects and residual variance, respectively. **G** is the genomic relationship matrix calculated according to VanRaden (2008) using genome-wide SNP information as:

$$\mathbf{G} = \frac{\mathbf{WW'}}{2\sum p_i(1-p_i)}$$

where **W** is centered genotype matrix with element c_{ij} - 2p_i.

Additive genetic variance, residual variance and subsequently heritability were estimated with restricted maximum likelihood (REML) method and the average information algorithm (Gilmour et al. 1995).

Fitting random animal effect with the use of genomic relationship matrix prevents false-positives association due to population stratification and cryptic relationships between individuals and also increases the power (Yang et al, 2014). Therefore the above model should be proper for Holstein population that has strong family structure due to the widespread use of few top bulls each year.

Inflation or deflation in p-values due to stratification or family structure was assessed by genomic inflation factor (λ) and also visually inspected by quantile-quantile (Q-Q) plot. λ is calculated as the median of the χ^2 test statistics (1 degree of freedom) divided by its theoretical median under the null distribution (Devlin and Roeder, 1999). In order to adjust for multiple comparisons, false discovery rate was controled at 1 and 5% genome-wise leveles (Benjamini and Hochberg, 1995). GWAS and heritability estimation were carried out by snp1101 software (Sargolzaei, 2014).

5.3 Results

The dataset contained 45,187 SNPs and after applying quality control mechanisms the number of SNPs used for the final analyses was 43,283. Animals with deviating days in milk (>500dim) were removed from the total dataset (n=6). Mean and the corresponding standard deviations for both IgG and IgM isotypes are shown in Table 5.1. Heritability based on genomic information for isotype IgG was estimated at 0.13± 0.062 and for isotype IgM heritability was estimated at . 0.27± 0.076.

Table 5.1: Means and standard deviations for immunoglobulin isotypes IgG and IgM. Both with and without log-transformation for 681 cows.

Variable	Mean	Std dev.	Min	Max
IgG	0.32	0.25	0.02	1.87
IgM	1.11	0.34	0.38	2.69
IgG_log	-1.41	0.76	-4.19	0.63
IgM_log	0.06	0.32	-0.96	0.99

Significant associations (FDR < 5%) between markers and natural antibody isotype IgG were found. Isotype IgM did not show any significant associations (Figure 5.1). The genomic inflation factor for IgG and IgM was 0.99 and .96, respectively. Figures 3 and 4 shows Q-Q plots of test statistics. In total, 5 significant markers were found (FDR < 5%), associated with IgG binding KLH. All 5 significant associated SNP for isotype IgG were located on chromosome 21, BTA-21 (Figure 5.2). In addition 5 suggestive associations were found. The region including siginifiacnt and suggestive associations spans from base pairs 55,532,125 to 70,759,096 towards the telomeric

end of BTA21 and contains several genes which can be functionally linked to IgG binding KLH (Table 5.2).

Table 5.2: Statistics on significant (FDR < 5%) and suggestive SNPs located on BTA 21 for natural antibody isotype IgG. Significant association are marked in bold.

Pos.	Freq.	b-value	p-value	-log10(p)	Gene ID	SNP
55532125	0,351	-0,32615	1,58E-05	4,801796685	TGM5	rs41609670
55617452	**0,322**	**-0,37822**	**1,50E-06**	**5,823931904**	**TP53BP1**	**rs109198033**
62445122	0,086	0,581542	3,67E-06	5,435301986	TUNAR	rs109331630
66492068	0,591	-3,19871	5,71E-05	4,243070405	EML1	rs110218992
67963614	0,824	4,17546	2,81E-05	4,55148073	DIO3	rs109169947
68105539	**0,797**	**-4,39826**	**2,11E-06**	**5,676349893**	**DIO3**	**rs110585835**
68399787	0,364	-3,28557	2,99E-05	4,524070345	PPP2R5C	rs110476540
68683196	**0,496**	**3,56351**	**4,40E-06**	**5,356997644**	**HSP90A1 / WDR20**	**rs110976149**
69340662	**0,207**	**-4,24405**	**5,97E-06**	**5,22423814**	**TRAF3**	**rs41638537**
69972343	**0,411**	**-3,58876**	**4,18E-06**	**5,378810212**	**PP1R13B**	**rs109922571**

Especially the genes TP53BP1, TRAF3 and HSP90AA1, because of their function , hint at a relevant relation with IgG levels. TP53BP1, tumor protein p53 binding protein 1, is essential for immunoglobulin class switch recombination, so it seems appropriate that this gene is highlighted in relation to IgG levels , whereas no effect is found for IgM levels. TRAF3,TNF receptor associated factor 3, is an important immune regulator. TRAF3 positively controls type I interferon production but negatively regulates mitogen-activated protein kinase (MAPK) activation as well as the alternative nuclear factor-κB (NF-κB) pathway. TRAF3 is important in B cells in signaling pathways activated by TNFR or Toll-like (TLR) receptor family members and thus control among others cytokine production and cell survival. (Häcker et al. 2011, Hildebrand et al. 2011). The WDR20, WD repeat domain 20, gene is implicated in the control of deubiquitination activity of different proteins important in cellular signaling pathways, e.g the P53/ Mdm and NF-κB pathway. Deubiquitination removes ubiquitin groups of proteins and thereby saves them for proteasome-mediated degradation or alters the activity of such proteins, while ubiquitination can target proteins for degradation. In the case of TRAF3 specific addition of ubiquitination at K48 (lysine 48) targets TRAF3 for degradation, thus enabling the downstream activation of NF-κB through I κB and p50and p65.
HSP90AA1, heat shock protein 90kDa alpha family class A member 1, is located close to WDR20 and is involved in control of the balance of humoral and cellular immunity by controlling the presentation of exogenous antigen (Li et al. 2012).

Furthermore a cluster of immunoglobulin heavy chain genes, as well as D region genes is located upstream toward the telomeric end of BTA21 at a distance of around 1,5 Mb. These genes all point to the regulation of B cells which are producing the antibodies studied. Although the log p values range only from 4- 5,8, the aforementioned genes can all be functionally linked to the control of the level of IgG binding KLH .

One significant SNP is located in PPP2R5C, protein phosphatase 2, regulatory subunit B', gamma. PPP2R5C is a phosphatase that operates by dephosphorylating multiple proteins in signaling pathways or biological processes (Cheng et al. 2015).

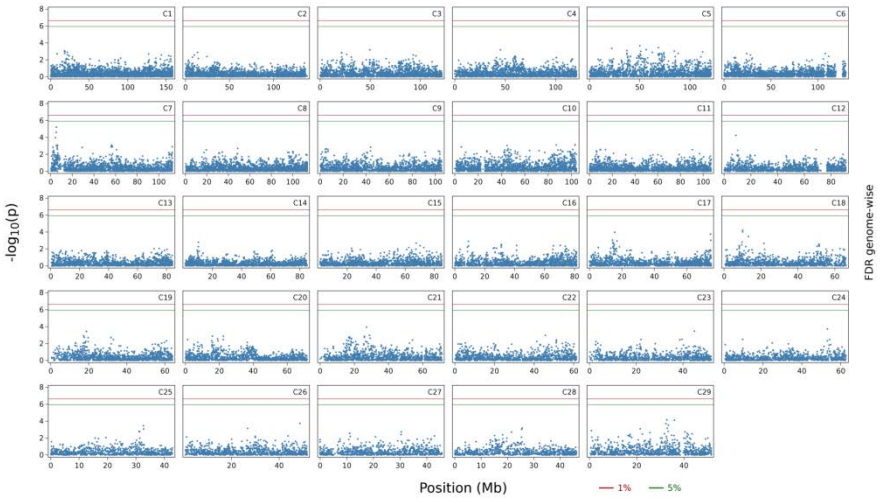

Figure 5.1: Distribution of -Log10 P-values from single SNP analyses for natural antibody isotype IgM binding KLH, for every chromosome. The red line indicates FDR rate of 1% and green line indicates FDR rate of 5%.

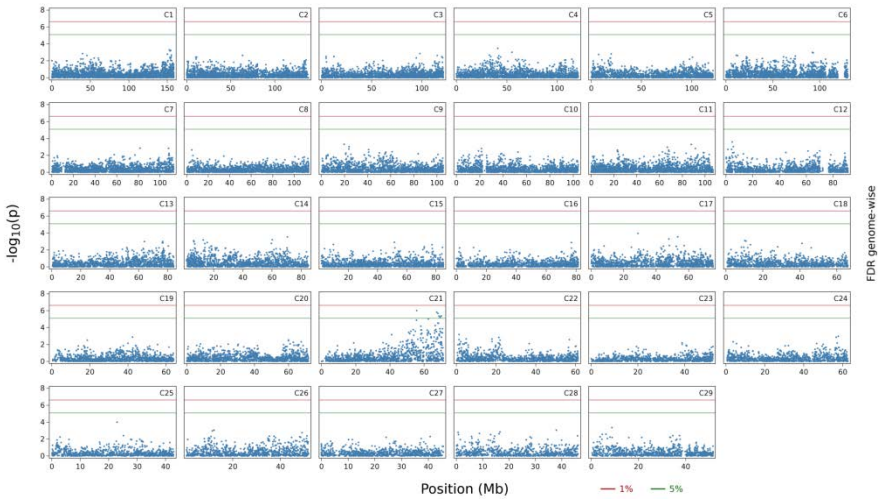

Figure 5.2: Distribution of -Log10 P-values from single SNP analyses for natural antibody isotype IgG binding KLH, for every chromosome. The red line indicates FDR rate of 1% and green line indicates FDR rate of 5%.

5.4 Discussion

5.4.1 Association analysis

The present study aimed to identify genomic regions involved in regulation of natural antibody levels, using cow data. Heritability was estimated with REML method using genomic relationships between individuals. Heritability estimate of IgM was 0.27, which was almost twice as h^2 of IgG. However, standard error of heritability estimate for both traits was relatively high due to small sample size. In this study no significant associations were found for isotype IgM even though the heritability of this trait was relatively high. This may indicate that underlying genetic architecture of IgM is more polygenic with no outstanding major genes. For Isotype IgG significant associations were found on chromosome 21 (BTA-21). For both traits, several peaks across genomes were observed that were not significant. These suggestive peaks should be further investigated in a bigger sample size.

In order to check for existence of population stratification or family structure, the GWAS study was repeated without including the animal random effect (the results were not shown). Genomic inflation factors (λ) were 1.25 and 1.44 for IgG and IgM, respectively, showing severe inflation in p-values. After properly correcting for

population stratification and family structure, λ should be close to 1 (Devlin and Roeder, 1999). Incorporating the full genomic covariance structure between individuals by fitting the random additive effect in th emodel resulted in λ close to 1 for both traits (.99 for IgG and 0.96 for IgM; Figures 5.3 and 5.4).

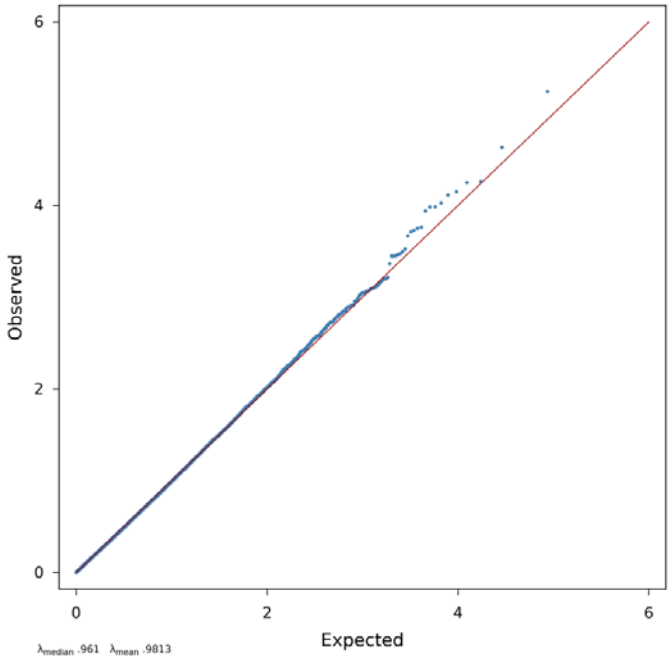

Figure 5.3: Q-Q plot of –Log10 p-values for natural antibody isotype IgM

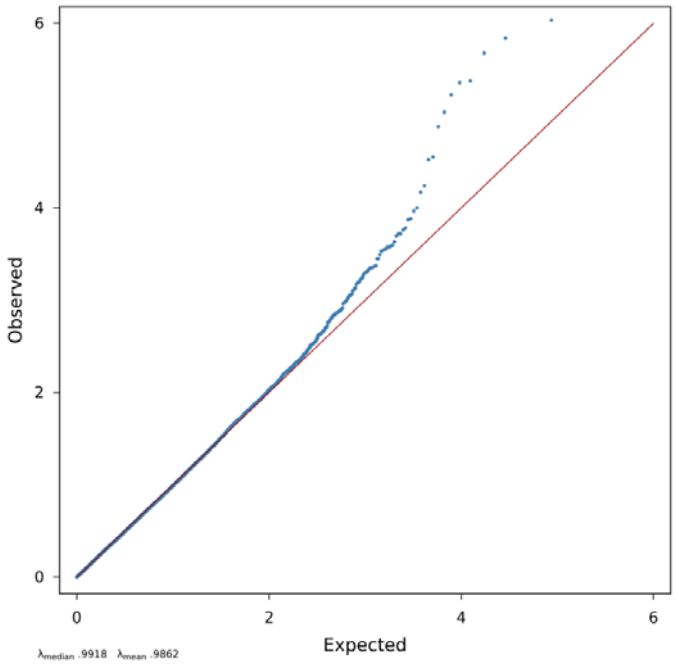

Figure 5.4: Q-Q plot of –Log10 p-values for natural antibody isotype IgG

5.4.2 Natural antibodies

The present study included natural antibodies of both isotype IgG and IgM, binding KLH. Natural antibodies are considered as the humoral part of the innate immune system (Baumgarth et al., 2005). The model antigen KLH is assumed to be not present in the common daily environment of dairy cows, and can therefore be assumed to be a naive antigen. Natural antibodies binding to naive antigens are, therefore, presumed to be the product of innate ability and reactivity in this study. Variation in levels of natural antibodies is caused by both genetic and environmental factors. To correct for environmental variation, herd was included in the model as variation exists in farm management, housing systems, infection pressure and feeding regime.

Natural antibodies are thought to be an indicator of natural resistance. However, few studies reported on relations between natural antibodies and diseases/resistance in dairy cattle. Ploegaert et al. (2010) reported a protective role

of antibodies against mastitis and an elevated level of somatic cell count. Moreover, natural antibodies are variable between individual cows and are under substantial genetic control (Wijga et al., 2013, Thompson-Crispi et al., 2013 and de Klerk et al. 2015). These findings, together with the ability of early protection and poly-reactivity of natural antibodies, make them potentially interesting parameters for aid in genetic selection for disease resistance. However, the precise role of natural antibodies in dairy cow disease resistance remains subject for further study.

5.4.3 Candidate genes

In this GWAS significant and suggestive associations between markers and natural antibody isotype IgG were found on chromosome 21 (BTA-21) (Table 5.2). On this chromosome some immunologically interesting genes are located within the significant associated genomic region of BTA21: TP53BP1,TRAF3, HSP90AA1, WDR 20, PPP2R5C, Ig heavy chain.

Immunoglobulin heavy chain genes and TP53BP1

Immunoglubulin heavy constant epsilon (IgHE) is located between 71468299 and 71473977 basepairs on BTA-21. Also the IgM and IgG2a heavy chain genes are located in the same region on BTA21 as well as several D and V segments.

In this study the closest significant SNP was found at 70,759,096 base pairs (approximately 700kb difference). The Immunoglobulin heavy chain genes are involved in class switching to enable the shift to the different functional characteristics of the different heavy chain classes M, G, A and E. Since in this study we observed significant SNP for IgG and not IgM binding KLH it is meaningful that TP53BP1 was showing a significant signal. This gene is controlling the class switch from IgM to IgG (Bothmer et al., 2011). Also it is necessary that TP53BP1 is in range of the genes to be switched, actually the heavy chain genes are located at about 1,5 Mb distal to TP53BP1. In man and mouse this range is smaller 0,7 Mb, but that might be tolerated by the recombination machinery.

TRAF3

TRAF3 encodes for protein TNF receptor-associated factor 3 and is responsible for the regulation of the effector function of regulatory T-cells and humoral immune responses. In B cells TRAF3 is involved in signal transduction of CD40 and BAFFR, in addition to TLR signaling (Häcker et al., 2011; Hildebrand et al., 2011). In conjunction with WDR20 which is involved in deubiquitination this generates

control over the P53/ Mdm and NF-κB pathway. It seems relevant in the context of the IgG levels that TRAF3 and WDR20 are highlighted in our analysis.

HSP90AA1

Heat shock protein 90 (HSP90) is a molecular chaperone required for efficient antigen presentation and cross-presentation (Li et al., 2012).

PPP2R5C

PPP2R5C, protein phosphatase 2, regulatory subunit B', gamma. is a phosphatase that operates by dephosphorylating multiple proteins in signaling pathways or biological processes (Cheng et al., 2015). PPP2R5C is involved in tumor malignancies in humans and mice, but recently was shown to be linked to metabolic status of the liver in man and mouse (Cheng et al. 2015). Cheng showed that expression of PPP2R5C is regulated in response to nutritional and metabolic status. Interestingly high KLH IgG and IgM levels in cows, during the last stage of pregnancy, have been linked to the risk to suffer from (severe) negative energy balance after parturition.

Interestingly, in a study by Wijga et al. (2013b), suggestive associations (FDR=20%) for isotype IgG binding KLH were found for chromosome 3, 21 and 25. Similar to our findings, the significant associations (FDR = 5%) in the study of Wijga et al., for isotype IgG, were found on chromosome 21, but only when binding to peptidoglycan (PGN). Differences between the study of Wijga et al. (2013) and current study might be explained by the population studied (Dutch versus Canadian Dairy cattle), numbers of animals studied, use of antigen (KLH vs PGN), and, Wijga et al. (2013b) measured antibodies in milk samples of dairy cows, whereas in the present study antibodies were measured in blood samples of the cows. However, regardless of the differences of study design, both studies report on interesting associations of IgG with genomic regions on chromosome 21.

This research provides insight in genetic control of natural antibody levels. However, more insight should be obtained on relations between natural antibodies and diseases. The GWAS performed in this study contained 681 cows. The detection power of the GWAS will likely be substantially increased when the number of animals studied is enlarged. However, with the amount of animals included in this study some interesting genomic regions were detected. An independent study is required for confirmation of the results obtained in this study. This was the first GWAS for natural antibody measured in blood in dairy cattle and

suggests it may be possible to include natural antibody traits, when their full function is known, in genomic selection.

5.5 Acknowledgements

This research was funded by grants to B.A. Mallard from the Semex Alliance, a collaborative research and development grant from the Natural Sciences and Engineering Research Council (NSERC) and the DairyGen Council of the Canadian Dairy Network (CDN). The authors' acknowledge the members of Dr. Mallards involved in the immune response sampling, Shannon Cartwright and Dr. Julie Schmied for technical laboratory advice, and Dr. Filippo Miglior (CDN) for providing the pedigree.

5.6 References

Benjamini, Y. and Y. Hochberg. 1995. Controlling the false discovery rate – A practical and powerful approach to multiple testing. J. of the Royal Statistical Society series B-methodological. 57:289-300.

Banos, G., E. Wall, M. P. Coffey, A. Bagnall, S. Gillespie, G. C. Russell, and T. N. McNeilly. s2013. Identification of immune traits correlated with dairy cow health, reproduction and productivity. PLoS One 8(6):e65766.

Baumgarth, N., J. W. Tung and L. A. Herzenberg. 2005. Inherent specifities in natural antibodies: a key to immune defence against pathogen invasion. Springer Semin Immunopathol. 26: 347-362.

Boes, M. 2000. Role of natural and immune IgM antibodies in immune responses. Mol. Immunol. 37:1141-1149.

Bothmer, A., D. F. Robbiani, M. Di Virgilio, S. F. Bunting, I. A. Klein, N. Feldhahn, J. Barlow, H. Chen, D. Bosque, E. Callen, A. Nussenzweig and M. C. Nussenzweig. 2011. Regulation of DNA end joining, resection, and immunoglobulin class switching recombination by 53BP1. Molecular Cell 42:319-329.

Carlèn, E., E. Strandberg and A. Roth. 2004. Genetic parameters for clinical mastitis, somatic cell score, and production in first three lactations of swedisch holstein cows. J. Dairy Sci. 87:3062-3070.

Casali, P. and E. W. Schettino. 1996. Structure and function of natural antibodies. Current topics in Microbiol. and Immunol. 210:167-179.

Cheng Y. S., O. Seibert, N, Klöting, A. Dietrich, K. Straßburger , S. Fernández-Veledo. 2015. PPP2R5C Couples Hepatic Glucose and Lipid Homeostasis. PLoS Genet 11(10): e1005561.doi:10.1371/journal.pgen.1005561.

De Klerk, B., B. J. Ducro, H. C. M. Heuven, I. Den Uyl, J. A. M. Van Arendonk, J. J. Van der Poel. 2015. Phenotypic and genetic relationships of bovine natural antibodies binding keyhole limpet hemocyanin in plasma and milk. J. Dairy Sci. 98 (4):1-7.

Devlin, B. and K. Roeder. 1999. Genomic control for association studies. Biometrics 55, 997-1004.

Gilmour, A. R., R. Thompson and B. R. Cullis. 1995. Average information REML: An efficient algorithm for variance parameters estimation in linear mixed models. Biometrics 51, 1440–1450.

Häcker H., P. H. Tseng and M. Karin. 2011. Expanding TRAF function: TRAF3 as a tri-faced immune regulator. Nat Rev Immunol. Jun 10;11(7):457-468.

Harmon, R. J. 1994. Physiology of Mastitis and Factors Affecting Somatic Cell Counts1. J. Dairy Sci.77(7):2103-2112.

Heriazon, A., K. A. Thompson-Crispi, B. N. Wilkie, W. Mathes-Sears, M. Quinton, and B. A. Mallard. 2009. Antibody to ovalbumin and delayed-type hypersensitivity to Candida albicans and myobacteria in lactating Holstein cows using Quil A or Freud's complete adjuvant. Vet. Immunol. Immunopathol. 127 (3-4):220-227.

Hernandez, A., N. Karrow, and B. A. Mallard. 2003. Evaluation of immune responses of cattle as a means to identify high or low responders and use of a human microarray to differentiate gene expression. Genet. Sel Evol. 35 Suppl 1:S67-S81.

Hildebrand J. M., Z., Yi, C. M. Buchta, J. Poovassery, L. L Stunz, G. A. Bishop. 2011. Roles of tumor necrosis factor receptor associated factor 3 (TRAF3) and TRAF5 in immune cell functions. Immunol Rev. 244(1):55-74. doi: 10.1111/j.1600-065X.2011.01055.x.

Kohler, H., J. Bayry, A. Nicoletti and S. V. Kaveri. 2003. Natural autoantibodies as tools to predict the outcome of immune response? Scandinavian J. of Immunol. 58:285-289.

Lin WW, B. S. Hostager, G. A. Bishop. 2015. TRAF3, ubiquitination, and B-lymphocyte regulation. Immunol Rev. Jul;266(1):46-55. doi: 10.1111/imr.12299

Li, Y., S. Li, M. Hoshino, R. Ishikawa, C. Kajiwara C, X. Gao, Y. Zhao, S. Ishido, H. Udono, J. Y. Wang. 2012. HSP90α deficiency does not affect immunoglobulin gene hypermutation and class switch but causes enhanced MHC class II antigen presentation. Int Immunol. Dec;24(12):751-8. doi: 10.1093/intimm/dxs076.

Ochsenbein, A. F. and R. M. Zinkernagel, 2000. Natural antibodies and complement link innate and acquired immunity. Immunol. Today 21 (12):624-630.

Ploegaert, T. C. W. 2010. Relation of natural antibodies with risk for mastitis and high somatic cell count in Dutch Holstein Friesian cows. Chapter 6, Page 81-92 in Phd Thesis: Parameters for natural resistance in bovine milk. Vol. PhD. Wageningen University, Wageningen, The Netherlands.

Ploegaert, T. C. W., S. Wijga, E. Tijhaar, J. J van der Poel, T. J. G. M. Lam, H. F. J. Savelkoul, H. K. Parmentier, J. A. M. van Arendonk. 2010b. Genetic variation of natural antibodies in milk of Dutch Holstein-Friesian cows. J. Dairy Sci. 93: 5467-5473.

Ploegaert, T. C. W., E. Tijhaar, T. J. G. M. Lam, A. Taverne-Thiele, J. J. van der Poel, J. A. M. van Arendonk, H. F. J. Savelkoul, H. K. Parmentier. 2011. Natural Antibodies in bovine milk and blood plasma: variability among cows, repeatability within cows, and relation between milk and plasma titers. Vet. Immunol. Immunopathol. 144: 88-94.

Pryce, J. E., R. J. Esslemont, R. Thompson, R. F. Veerkamp, M. A. Kossaibati, G. Simm. 1998. Estimation of genetic parameters using health, fertility and production data from a management recording system for dairy cattle. An Sci 66:3 577-584.

Rupp, R. and D. Boichard. 1999. Genetic parameters for clinical mastitis, somatic cell score, production, udder type traits and milking ease in first lactation Holsteins. J. Dairy Sci 82:2198-2204.

Rupp, R. and D. Boichard. 2003. Genetics of resistance to mastitis in dairy cattle. Vet. Res. 34:671-688.

Sargolzaei, M. 2014. SNP1101 User's Guide. Version 1.0.

Sargolzaei, M., J. P. Chesnais and F. S. Schenkel. 2014. A new approach for efficient genotype imputation using information from relatives. BMC Genomics, 15:478.

Thompson-Crispi, K. A. and B. A. Mallard. 2012a. Type 1 and type 2 immune response profiles of commercial dairy cows in 4 regions across Canada. Canadian Journal of Veterinary Research 76 (2):120-128.

Thompson-Crispi, K. A., B. Hine, M. Quinton, F. Miglior and B.A. Mallard. 2012b. Short communication: Association of disease incidence and adaptive immune response in Holstein dairy cows. J. Dairy Sci. 95:3888-3893.

Thompson-Crispi, K. A., A. Sewalem, F. Miglior and B.A. Mallard. 2012c. Genetic parameters of adaptive immune response traits in Canadian Holsteins. J. Dairy Sci. 95:401-409.

Thompson-Crispi, K. A., F. Miglior, and B. A. Mallard. 2013a. Genetic parameters for natural antibodies and associations with specific antibody and mastitis in Canadian Holsteins. J. Dairy Sci. 96(6):3965-3972.

Thompson-Crispi, K. A., F. Miglior and B. A. Mallard. 2013b. Incidence of clinical mastitis among Canadian Holsteins classified as high, average, or low immune responders. Clin. and Vaccine Immunol. vol20 (1)106-112.

Van Knegsel, A. T. M., G. De Vries Reilingh, S. Meulenberg, H. Van den Brand, J. Dijkstra, B. Kemp and H. K. Parmentier. 2007. Natural antibodies related to energy balance in early lactation dairy cows. J. Dairy Sci. 90, 5490–5498.

Van Knegsel, A. T. M., H. Hostens, G. de Vries Reiling, A. Lammers, B. Kemp, G. Opsomer and H. K. Parmentier. 2012. Natural antibodies related to metabolic and mammary health in dairy cows. Preventive Veterinary Medicine 103:287-297.

VanRaden, P. M. 2008. Efficient methods to compute genomic predictions. J. dairy Sci. 91:4414-4423.

Wagter, L. C., B. A. Mallard, B. N. Wilkie, K. E. Leslie, P. J. Boettcher, and J. C. Dekkers. 2000. A quantitative approach to classifying Holstein cows based on antibody responsiveness and its relationship to peripartum mastitis occurrence. J. Dairy Sci. 83(3):488-498.

Wijga, S., H. Bovenhuis, J. W. M. Bastiaansen, J. A. M. van Arendonk, T. C. W. Ploegaert, E. Tijhaar and J. J. van Poel. 2013. Genetic parameters for natural antibody isotype titers in milk of Dutch Holstein-Friesians. Anim. Genet. 44: 485-492.

Wiggans G., T. Sonstegard, P. VanRaden, L. Matukumalli, R. Schnabel, J. Taylor, F. Schenkel and C. Van Tassell. 2009. Selection of single-nucleotide polymorphisms and quality of genotypes used in genomic evaluation of dairy cattle in the United States and Canada. J Dairy Sci. 92:3431–3436.

Yang J, N. A. Zaitlen, M. E. Goddard, P. M. Visscher, A. L. Price. 2014. Advantages and pitfalls in the application of mixed-model association methods. Nat Genet. 46(2):100-6.

6

General Discussion

6.1 Introduction

For manuy years breeding in dairy cattle has been mainly focused on production and milk quality traits. After generations of selection on these traits cows are able to produce a large amount of milk of good quality. Over time it has become clear that selection should not only be aimed at high milk production but also at health and welfare traits. Selection including health and longevity related traits aims to reduce incidence of mastitis and claw problems, to reduce the impact of negative energy balance, and to increase longevity. Increasing longevity of dairy cattle will be beneficial for the economics of the farmers, the animal welfare and overall sustainability. The main problem with selection on health or longevity traits is that phenotypic records for these traits are only available when a cow has already been diseased or culled. There is a need for traits that better reflect or predict the longevity of a cow at an early age. Longevity is highly correlated with disease resistance and as a result studies have focused on finding biomarkers that can reflect or predict the overall disease resistance of a cow. In this respect disease includes both infections as well as (metabolic) disorders. It is, however, difficult to distinguish cause and consequences and relationships between metabolic disorders and infections. Somatic cell count is an example of an applied biomarker for health status as it mirrors the udder health of a cow (Harmon et al, 1994; Bradley 2002; Samoré et al. 2003). This biomarker is used in selection to decrease incidence of mastitis, one of the most frequent and costly diseases in dairy cows (Halasa et al., 2007). However, mastitis is not the only important disease in cows. Therefore, additional biomarkers are needed to improve the prediction of the overall health and longevity of a cow. Since antibodies are highly related to disease resistance both with respect to infections and metabolic disorders (Baumgarth et al., 2005; Lutz and Miescher 2008; Lutz et al., 2009) and consequently to longevity, antibody levels and antibody isotypes could be good measurable traits to predict the longevity potential of a cow. Antibodies were suggested as an indicator for the metabolic state of a cow (van Knegsel et al., 2012) which is very important in maintaining a healthy energy balance.

The main goal of the research in this thesis was to evaluate the potential of using antibodies as a biomarker for longevity traits. In practice, measuring a biomarker in milk rather than in blood is cheaper, less labor intensive and more animal friendly. However, the relationship between antibody levels and isotypes in blood and milk is not always clear. Therefore, it was important to know more about the relation between antibodies measured in blood and in milk. Another aim was to investigate

the relation between antibody levels and longevity in dairy cows. The relation between antibodies and longevity has a large impact on the value of antibodies as predictors for longevity and its value for breeding programs focusing on improved longevity. Finally this thesis aimed to detect genomic regions that are responsible for antibody regulation. The results reported in this thesis showed that: **Chapter 2**) Antibodies measured in milk and blood are phenotypically different traits but are genetically highly related; **Chapter 3**) Phenotypically, antibody levels measured in milk are negatively related with longevity, since first lactation cows with a lower antibody level on average have a longer productive life; **Chapter 4**) The prospective longevity of an animal, described by estimated breeding values is negatively related with antibody levels. First lactating cows with low antibody levels have higher estimated breeding values for longevity; **Chapter 5**) A genome-wide-association study revealed genomic regions that are responsible for antibody levels regulation, especially regions on chromosome BTA-21 are of interest. However, in this thesis it could not be addressed whether higher or lower levels of antibodies reflect either an activation status of the immune system, or the potential to respond to disease inducing agents with possible negative consequences for longevity.
The aim of this general discussion is to put the results of previous chapters in a broader perspective. Therefore, in this final chapter the focus is on the biology and definition of antibodies, in order to get a better understanding of this trait. Also the relation between different antibody traits and longevity is discussed. Lastly, opportunities for future dairy breeding based on antibody traits is discussed.

6.2 Antibody definition

Most antibodies bind to PAMPs (pathogen-associated molecular patterns) that represent antigens that are shared by microbes and serve as targets for identification of microbes by the innate or adaptive immune system (Kohler et al., 2003). Antibodies are immunoglobulins which functions include opsonization, neutralization and complement activation (Janeway et al., 2001). In this thesis different (iso-)types of antibodies were measured; IgM, IgG and IgA binding different important PAMPs: lipopolysaccharide (LPS) present on gram-negative (entero)bacteria, such as *E. coli* or *Salmonella spp*; lipoteichoic acid (LTA) present on gram-positive bacteria such as *Staphylococcus aureus*; peptidoglycan (PGN), present on gram-negative bacteria as well as gram-positive bacteria, and keyhole limpet hemocyanin (KLH), derived from the *Megathura crenulata*. Keyhole limpet hemocyanin was chosen as antigen, because it is assumed to be a naive antigen (Star et al. 2007). This thesis showed that the IgM isotype is the isotype with the

highest heritability, indicating that genetic factors have highest contribution to variance of this trait (**Chapter 2 and Chapter 5**). When looking at phenotypic traits, we found that traits that are more influenced by environmental factors, in this case IgG and IgA, show more significant relations with phenotypic longevity **(Chapter 3)**. Only in the case of antibodies binding LTA, a significant relation was found between longevity and isotype IgM. **In Chapter 4**, the relation between antibody levels and genetic merit for longevity was estimated. Genetic merit for longevity was based on estimated breeding values (EBV's) for productive life. EBV's including early predictors (=indirect longevity) and EBV's based on productive life records only (=direct longevity) were considered in this study. Some significant relations were found between longevity (direct and indirect) and IgG, IgA and IgM. In case of estimated breeding values for direct longevity, the only significant relation that remained was the one between longevity and isotype IgM binding KLH. The latter is in line with the fact that IgM is the most heritable trait. In this thesis antibodies binding KLH were assumed to be natural antibodies. To our knowledge no earlier results were published on innate markers for longevity/survival. It is an interesting finding that exclusively isotype IgM binding KLH, which we assume is a part of the innate immune system, had a significant relation with longevity.

6.2.1 Natural antibodies vs specific antibodies

There are two major types of antibodies, natural antibodies and specific antibodies. Natural antibodies are defined to be present in individuals that have not encountered the antigen before, whereas specific antibodies reflect response or memory to a specific antigenic challenge, infection or vaccination. This thesis focused mainly on natural antibodies, especially antibodies binding keyhole limpet hemocyanin (KLH). These antibodies were assumed to be natural antibodies since KLH is derived from the *Megathura crenulata*, a sea snail that lives at the bottom of the ocean. Therefore KLH is supposed to be a naive antigen for dairy cows as it is highly likely that cows have never been exposed to this protein. Also, cross reactivity between KLH and other antigens has not been reported to the best of our knowledge. Additionally, this thesis also monitored antibodies which bind lipopolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglycan (PGN). It is known that these structures are present on the cell membranes of bacteria, and are therefore more likely to be encountered in a dairy cow's environment. Therefore we presume that cows might have been in contact with these bacterial membrane antigens before we measured the antibodies in our experiments. The

antibodies measured in response to these structures can be assumed to be specific antibodies generated in response to previous or continuous exposure.

6.2.2 Natural antibodies

Natural antibodies in man and mice are produced by B1 type B-cells and are present in large amounts in the blood and mucosal secretions. Natural antibodies are responsible for direct neutralization of pathogens, complement-mediated lysis and prevention of bacterial and viral spreading (Ochsenbein and Zinkernagel, 2000), and are mostly directed against non-self-antigens (Lutz et al. 2009). Natural antibodies can also be present after (immune) stress of the host and be directed against self-antigens (Cheng and Chamley, 2008). The current view is that natural antibodies (mainly from the IgM isotype), function as barrier against infections and as adjuvant for subsequent specific immune responses (Balsari and Caruso, 1997). In humans, deregulation of antibodies (class switching; IgM to IgG or IgA), can be a basis for (auto) immune diseases and chronic inflammation (Ehrenstein and Notley, 2010). Natural antibodies play an important role in the prevention of infection and restoration of the homeostasis after infection and stress. Therefore, natural antibodies provide a key protection during the period between onset of infection and the emergence of the adaptive immune response. Consequently, natural antibodies constitute a potentially important humoral component of innate immunity (Casali and Notkins, 1989). Natural antibodies can, therefore, be considered as an important trait when looking at natural resistance.
In dairy cows Ploegaert et al. (2010b) showed that natural antibodies level are potential predictors for diseases for individual cows. In earlier studies on relations between natural antibodies and diseases, higher natural antibodies were found to be favourable (Boes et al., 1998; Star et al., 2007; Banos et al. 2013; Thompson-Crispi et al., 2013a; Machado et al. 2014). Given the above mentioned characteristics of natural antibodies It seems reasonable to assume that natural antibodies can be considered to be factors that are involved in natural resistance. In the next sections, I will discuss more on the interpretation of the differences in results regarding the relation between natural antibody levels and health and longevity.

6.2.3 Specific antibodies

Specific antibodies in man and mice are produced by B2 type B-cells and are part of the adaptive immune system. Specific antibodies are mainly produced in response to antigens that activate antigen presenting cells and T cells. Adaptive responses

involve memory and specific activation of B2 type B-cell receptors, resulting in a response (increasing levels and affinity maturation) which is delayed as compared to the barrier function provided by natural antibodies (Baumgarth et al., 2000). Higher levels of NAb may result, however, in lower levels and lower affinity of specific antibodies (SpAb) and the other way around (Parmentier et al. 2008). Figure 6.1 shows the differences in response mechanism between natural antibodies and specific antibodies when an antigen is entering the body. Specific antibodies only bind to specific antigens they can recognize, whereas natural antibodies have a broad binding spectrum with lower binding affinity that enables NAb to bind to a large variety of unknown antigens as well. Natural antibodies, therefore, can provide an immediate and broad barrier as opposed to specific antibodies as a first line of defence. Whether SpAb subsequently reside from the initial NAb population is, however, still a matter of debate.

6.2.4 Comparison natural antibodies and specific antibodies

In this thesis both natural- (KLH) and presumably specific antibody (LPS, LTA, PGN) levels showed a negative relation with longevity (**Chapter 3 and Chapter 4**). Natural resistance is an important trait, since it is responsible for protecting individuals against invading pathogens, but it also regulates overall health. This makes it interesting to see that in **Chapter 4,** the only significant relation that remains, is the one between longevity and natural antibody isotype IgM. According to our findings, the antibody isotype IgM, is a heritable trait that gives information on the potential of an animal to live a longer life. IgM is the first membrane bound antigen receptor on the pool of maturating B cells. Antigen stimulation and T-cell helper cells are usually required for isotype switching to IgG or IgA suggesting that the

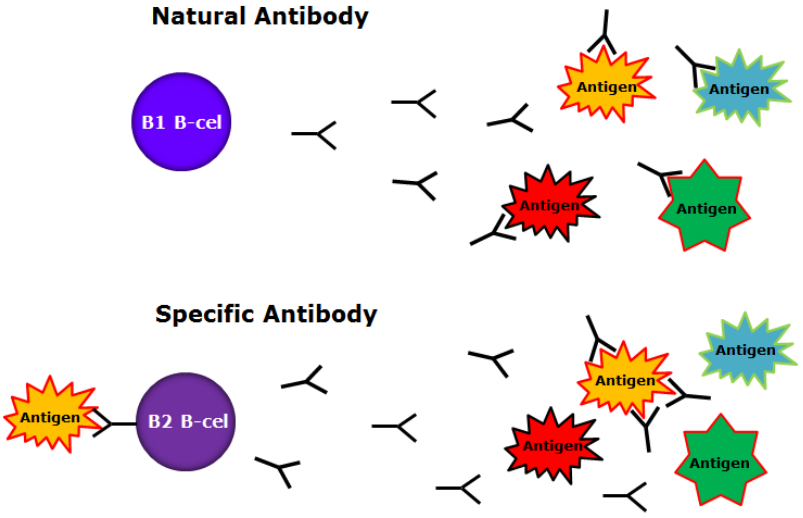

Figure 6.1: Difference between natural- and specific antibody response mechanism when responding to antigens. Showing natural antibody produced by B1 B cells and specific antibody produced by B2 B cells (Christine van Altena).

latter two isotypes reflect immune activation. Specific antibodies on the other hand, are sometimes hard to distinguish from natural antibodies, since ELISA tests in the lab, cannot determine easily whether antibodies are produced in response to known antigens (based on antigenic stimulation or memory information), or as the levels of natural antibodies circulating as part of first line defence.

B2 B-cells produce individual specific antibodies, have distinct receptors, which makes every B cell unique; every B2 B-cell can respond to different antigens. At first contact (primary response), a sufficient immune response needs to be started (which takes about a week) before specific antibodies will be produced. Additionally, memory cells are created. Memory cells are involved in secondary response, so a faster and more efficient response can be activated as soon as there is contact with the recognized antigen. Natural antibodies, on the contrary are especially important in the first phase of an immune response. The immune system needs to identify an intruder. In case of new intruders, sometimes it can take too long before specific antibodies are produced. Bacteria can divide every 20 minutes, so a fast response is required. Therefore, the fast responding natural antibodies are crucial for protection of individuals. Levels of specific antibodies, consequently, give more insight in what an animal has suffered from so far (bacteria and pathogens

encountered), natural antibodies on the other hand give more information on the potential of a cow to react to something new.
Based on our findings and literature, natural antibodies (especially isotype IgM) thus may be a more reliable (genetic) predictor for longevity than specific antibodies.

6.2.5 Milk antibody vs blood antibody

Antibodies can be measured in both secretions such as milk and in blood (**Chapter 2**). The advantage of measuring antibodies in milk instead of blood is that analysis of antibody levels in milk is more easily feasible and cheaper compared to measuring antibody levels in blood. This study showed that there is a high genetic correlation (~0.80), between the same antibody measured in milk and blood (**Chapter 2**). The phenotypic correlation between the same antibody measured in milk and blood, however, is lower ~0.40 (**Chapter 2**). A factor that might contribute to the lower phenotypic correlation is the variation in amount of milk produced by cows. However, additional analysis revealed that correcting for the amount of sample milk did not influence the individual antibody levels. This suggests that the effect of variation in milk production is negligible.
Antibodies in blood are either systemic and transported to places where needed or reflect ongoing local immune responses. The low phenotypic correlation between antibodies in blood and milk may be caused by 3 different aspects. Firstly, antibodies are either selectively or passively transferred from blood to milk through the mammary epithelium, mostly by diffusion (Östensson and Lun 2008). Secondly, the excess of non-necessary antibodies need to be excreted by the body, for instance via the udder. Finally, the existence of a local source of IgM producing cells in the mammary tissue was indicated, which initiates IgM production in response to infection (Östensson and Lun 2008). These different processes of antibody transport, and local production, make it reasonable that antibodies measured in milk and blood should be interpreted as phenotypically different traits. However, if antibody levels in milk correspond to some (known) extent to antibody levels in blood, this could be useful as an explanatory factor, especially when genetic correlations are present.
Antibody levels can be more easily obtained from milk, in combination with regular milk quality control, which makes them a good parameter for monitoring health status of a cow. Additionally, it would be possible to measure antibody levels on a very regular basis, for example with help of the innovative robot milking systems that are used nowadays. Repeated measurement of antibody levels makes it

possible to make predictions of longevity more accurate. As a warning system, repeated measurement of antibodies in milk could also help to monitor the health status of a cow along the milk trajectory that could help the farmer to detect health problems as early as possible or alternatively provide selection tools for breeders.

6.2.6 Antibodies vs somatic cell counts

Somatic cell count is a commonly used biomarker for udder health in dairy cattle, both for breeding and herd management. Somatic cell count has a relatively high (0.70 - 0.72) genetic correlation with mastitis (Rupp and Boichard, 2003; Carlèn et al., 2004) and is therefore mostly used as an indirect measure of mastitis (Mark et al., 2002). In literature, it is reported that antibodies are not only related to mastitis but also to other health traits, like claw health and metabolic diseases, especially pre- and postpartum (Van Knegsel et al., 2007 & 2012; Banos et al, 2013). These relations were also investigated within the "WeerbaarVee" project as described in chapter 6.3.1.

Heritability for average somatic cell count during lactation generally ranges between 0.10 and 0.17 (Pryce et al., 1998; Rupp and Boichard, 1999 & 2003; Carlèn et al., 2004). In this thesis (**Chapter 2 and Chapter 5**) and other studies (Ploegaert et al., 2010; Thompson-Crispi et al., 2013b; Wijga et al., 2013) it is concluded that levels of antibodies (depending on isotype and binding specificity) are more heritable than somatic cell count, with heritabilities ranging from 0.09 to 0.45. This makes antibodies a suitable trait for selection, provided that antibodies comprise variation as well.

Somatic cell counts were introduced as a selection trait for improved udder health in breeding programs. A higher amount of somatic cells is related to higher risk of mastitis (Sharma et al., 2011). However, too low levels of somatic cells are not preferable either (Suriyasathaporn et al., 2000). Somatic cells are part of the immune system, too low levels might be a risk for mastitis as well. For antibodies, likely also a certain minimum level of antibodies is needed to cope adequately with infection and, therefore, too low levels may be undesirable. However, too high antibody levels might be disadvantageous too as can be derived from the results of this study. This suggests that high antibody levels might be a risk for hypersensitivity or autoimmunity. Additionally, a more activated immune system takes more energy, which is disadvantageous for other body processes. Combining the pros and cons of too high and too low antibody level it seems necessary to aim for an optimum for antibody levels.

6.2.7 Variation of antibody levels

Antibody levels show a great amount of variation. A large part of this variation is caused by affinity and specificity maturation which is likely influenced by environmental factors like farm management, (antigen-)disease exposure and housing conditions. Within the project "WeerbaarVee" antibodies binding KLH (natural antibodies) were measured in both blood- and milk samples. Under different circumstances antibody levels measured in blood seemed to stay more or less stable over time (**Chapter 2**), whereas antibodies measured in milk were more responsive to individual cow traits like parity and lactation stage. Additionally, antibody levels are also influenced by season and feed-regimes. The largest impact of environmental factors was found for isotype IgG. For example, based on farm averages, farms with higher scores for good calf-housing and overall cow-comfort, had a lower mean IgG level.

In the ''WeerbaarVee'' project cows with high levels of natural antibody binding KLH levels were identified. Under supervision of a veterinarian modulations were considered per individual farm. Modulations such as optimisations of dry-cow management and feeding regimes decreased levels of IgM antibodies and disease incidence by a factor of 1.6. This suggests that 1) IgM antibodies were related with disease sensitivity post-partum, and 2) modulation of IgM levels is possible and consequently may impact metabolic disease sensitivity.

It is difficult to determine what antibody level, binding specificity and isotype are optimal for an individual cow. Most studies report the protective role of antibody levels; and found positive correlations with antibody levels and protection against different diseases (Van Knegsel et al., 2007 & 2012; Banos et al, 2013). Results of the above mentioned studies, however, were based on sampling of the immune system on specific moments. Other studies measured the kinetics or immune responses prior to and after immunization (Wagter et al., 2000; Thompson-Crispi et al., 2013a). In that case it is possible to determine differences in response to a certain antigen between different animals.

The results of this thesis indicate that the antibody levels may 1. reflect the temporary health status of a cow, or 2. reflect the potential pool of antibodies that provide immunity to a cow. With regard to the first, cows with higher antibody levels to LPS, LTA, or PGN, especially IgG antibodies might be fighting an infection or have health problems at the time of measurement. Such cows that chronically have high antibody levels to such bacterial antigens, could be 'problem' cows. Alternatively, chronically high (especially IgM) antibodies to naïve antigens such as KLH may indicate cows with a higher capacity or sensitivity potential to respond. A

distinction between temporary and chronically levels of antibodies and their isotypes and specificities is required to further improve the value of antibody levels as biomarker. The implication may be that a balanced antibody level (IgM and or IgG) is best for a cow. Too low or too high is unfavourable, meaning that a balanced antibody level reflects a cow with a balanced immune homeostasis, yet able to respond when challenged. What level of antibody stands for a well-balanced immune system, still needs to be further studied. For example it could be informative to measure base antibody levels at the earliest life stage possible; in newborn calves. Next antibody levels should be monitored during the rearing period of the calves to get more insight in how antibody levels develop.

6.3 Antibodies and longevity

The potential of antibody levels as biomarker was studied, since antibody levels are known to give information on different disease traits, and might therefore be related to longevity as well. In this thesis we found that lower levels of antibodies in young cows correlated to an increased chance to live longer compared to young cows that have higher antibody levels binding the antigens LPS, LTA, PGN and KLH(**Chapter 3** and **Chapter 4**). Whether this is also true for antibody levels binding other antigens or auto-antigens remains to be studied. Between the 10% animals with the highest values for antibodies and the 10% animals with the lowest values for antibodies in milk, a difference in productive days was found between ~47 and ~149 days, depending on the isotype and antigen. No earlier study on relations between antibodies and longevity in dairy cows was published. In poultry, studies on laying hens (12 elite pure bred lines) showed opposite antibody and survival correlations, compared to results reported in this thesis. Star et al. (2007) and Sun et al. (2011) found that higher antibody levels in blood are correlated with an increased survival period of laying hens. Comparison to the situation in laying hens might be questionable for several reasons. All chickens were sampled at the same age and on a single farm whereas cows were sampled at different ages and in different farms. One can debate that higher antibody levels offer increased protection to (certain) diseases, but one can also debate that low levels could indicate that an individual is fine and no disease is present. It is possible that the difference between cows and poultry is caused by a difference in the absolute level of natural antibodies. When levels in poultry are low, an improvement could explain a higher survival resulting from a better protection. Moreover, differences between cow- and chicken survival studies might be due to the difference in survival definition. Survival of dairy cows is a different trait compared to survival of

laying hens. Generally, laying hens die a 'natural dead', whereas dairy cows are in most cases culled for other reasons, like fertility-, infection-, or lameness problems, normally no causes of a 'natural death'.
Recently a relation was found between enhanced level of natural antibodies binding oxidized low density lipoprotein (LDL, (bad-cholesterol)) and a decreased risk of hepatic inflammation in mice. Interestingly, the ox-LDL antigenic component: phosphatidyl choline (PC) is present in gram-positive streptococci, and vaccination with the bacteria decreased hepatic inflammation (Bieghs et al., 2012). This suggests 1) relations between infectious agents and metabolic disorders might sometimes be counter-intuitive, and 2) the involvement of antibodies in the prevention of metabolic disorders as well as infections. In this respect the relationship between disorders (longevity) and antibodies binding LPS, LTA, or PGN should not be solely based on assumed specific antibody responses to infection, but increased metabolism should be considered as well.

6.3.1 Antibodies and health

An currently practised approach to breed for improved disease resistance is to select animals with an enhanced specific immune response (Wilkie and Mallard, 1999; Abdel-Azim et al. 2005; Mallard et al. 2011). In dairy cattle specific cell-mediated and antibody mediated immune responses have been used as indicator of immune response (Wagter et al. 2000; Cartwright et al., 2011; Hine et al., 2011; Thompson-Crispi et al. 2012). Dairy cattle with a higher immune response have been found to have a lower incidence of mastitis, metritis, displaced abomasum, retained placenta compared to cows with a lower immune response in the herd (Thompson-Crispi 2012a). No specific immune responses "sensu stricto" to specific antigens or vaccines were measured in the ''WeerbaarVee'' project, thus a relationship between NAb and 'real SpAb' could not be established.
Results from the ''WeerbaarVee'' project showed that especially natural antibodies measured in milk were associated with diseases, that are related to the initiation of the lactation (so-called postpartum diseases), like retained placenta, endometritis and ketosis. The incidences of these postpartum diseases were analyzed by two different methods; logistic regression and survival analysis. Using logistic regression, it was tested if animals that were at least 200 days in lactation, developed a disease in the 200 days after sampling. In the survival analysis it was estimated whether antibody level was associated with the risk of cows to develop a disease 60 days after calving. Both models were able to distinguish animals that had a high- or low risk to develop a postpartum disease.

The models were used to classify 33% of the cows as high-risk and 31% of these cows actually developed a disease in the following lactation. The analyses revealed that cows with a high level of antibodies were 2.7 times more likely to develop a disease (OR=95%, CI=2.2-3.3) compared to cows with low levels. Besides the association between milk natural antibodies and postpartum diseases, an association was found between milk natural antibodies and white line disease (claw disease). Animals with a higher level of IgM isotype binding KLH, were more likely to develop white line disease or a postpartum disease in their next lactation. Findings of ''WeerbaarVee'' at first sight seem to contradict with earlier studies, where it was found that lower levels of antibodies were associated with more diseases, like mastitis, metritis and metabolic disorders (Ploegeart et al., 2010b; Van Knegsel et al., 2012; Thompson Crispi et al., 2013a and 2013b). If we combine present and earlier findings; antibodies seem to protect against diseases, but a high level of antibodies seems to be an indication of a too strong immune system trigger. Also, in this thesis and the ''WeerbaarVee'' project, the predictive value of antibodies was mainly studied; i.e. what information can levels of antibodies give on the emergence of prospective diseases. In other studies relationships were studied of antibodies and immune parameters with diseases within the same period of time. Moreover, as mentioned before, this antibody-phenomenon shows a parallel with somatic cell count. Somatic cells (mainly leukocytes) are responsible for dealing with bacteria, so it is beneficial to have enough somatic cells to fight bacteria. Consequently, the higher the number of somatic cells, the higher the chance that the animal is suffering from mastitis at that very moment (Beaudeau et al. 1998; Rupp et al., 2000).

Other results from the ''WeerbaarVee'' project support the negative relation found between antibody levels and longevity (**Chapter 3 and Chapter 4**). In ''WeerbaarVee'', for every farm a Herd-Life-Index (HLI) was calculated. The HLI is a combination of mean age of animals present on the farm (in days), and the mean age of cows when being culled. Mean levels of natural antibody isotype IgG and IgM of first parity cows were determined for farms with a high HLI and for farms with a medium HLI. To test the hypothesis if natural antibody levels differ between high- and medium ranked HLI farms, natural antibodies were beforehand corrected for effects like parity, lactation stage and farm.

For both isotypes IgG and IgM, we observed a difference between the 2 farm groups, but only a significant difference was found for isotype IgG.

6.3.2. Antibodies applied in breeding

Whether antibodies are suitable to be applied in breeding depends on the genetic variance and the genetic correlation with traits of the breeding goal. Genetic variance of antibodies have been estimated in previous studies (Wijga et al., 2013 ; Ploegaert et al., 2010b). Relationship to the breeding goal trait, longevity, have been demonstrated in **Chapter 3** and **Chapter 4** of this thesis. For incorporating into a breeding program the genetic correlations of antibodies with longevity are required.

Genetic correlations were estimated based on the same dataset as used in **Chapter 4**, i.e. records on a set of antibodies of 1453 first parity cows.

We decided to estimate correlations between breeding values of bulls for antibodies and longevity as a proxy of the genetic correlations. The breeding values of longevity were supplied by CRV and were from their routine breeding values estimation, implying that they are much more accurate than breeding values we estimated from the 1453 phenotypes of our dataset. Breeding values both for direct and indirect longevity were provided. Univariate animal models were applied to the antibody phenotypes to estimate breeding values for bulls (n=97) based on the phenotypic observations on their daughters. The animal models was as follows:

$$Y_{ijklm} = \mu + b_1 \text{ x } DIM_i + b_2 \text{ x } AFC_j + BATCH_k + FARM_l + ANIMAL_m + e_{ijklm}$$

Where the response variable Y represented the different antibody binding different antigens (i.e. IgM-KLH, IgM-LPS, IgM-LTA, IgM-PGN, IgG1-KLH, IgG1-LPS, IgG1-LTA, IgG1-PGN, IgA-KLH, IgA-LPS, IgA-LTA, IgA-PGN), μ was the population mean, DIM was covariate describing the effect of days in milk, with regression coefficient b_1, AFC was covariate describing the effect of age at first calving in days, with regression coefficient b_2, BATCH was fixed effect of day of analysis (2 different days), For Ab binding to antigen LTA same fixed effects were used, except for 'BATCH', since LTA Ab were tested on 9 different days instead of only 2. Therefore, in this case BATCH was fixed effect of day of analysis (9 different days). FARM was random herd effect, distributed as $\sim\mathbf{N}(0,\mathbf{I}\sigma^2_h)$, ANIMAL was the random animal effect, distributed as $\sim\mathbf{N}(0,\mathbf{A}\sigma^2_a)$ and e was the random residual (distributed as $\sim\mathbf{N}(0,\mathbf{I}\sigma^2_e)$. Matrix I is the identity matrix and matrix A represents the additive relationships between animals.

Subsequently, Spearman correlation coefficients were estimated using PROC CORR of SAS®. The genetic correlations were approximated by applying the method of Calo (Calo et al., 1973) to the correlations between breeding values, to account for reliabilities of breeding values less than unity.

Results showed that the heritability estimates for antibody levels were in line with the heritability estimates as found by Wijga et al. (2013). The mean breeding values of antibody levels for sires are presented in Table 6.1. Breeding values were scattered around a mean close to zero for each antibody isotype binding different antigens, and were ranging from negative to positive breeding values. The range of negative to positive breeding values indicate that bulls can be selected that will reduce or increase antibody levels in its progeny. Average reliability of breeding values for antibody levels for these bulls were ca. 0.20-0.30 for IgG isotypes and ca. 0.45-0.55 for IgA and IgM isotypes.

Table 6.1 also contains the breeding values for direct and indirect longevity for bulls, as provided by CRV. Both breeding values showed large variation and ranged from -414 to +580 days for indirect longevity and only slightly lower values in case of direct longevity (-411 and +572 days resp.). Reliabilities of longevity breeding values all were higher than 83%. The correlation between breeding values for direct and indirect longevity was 0.99 and the behaviour in relations to the antibody levels appeared to be similar. In the remainder of this section any further distinction between direct and indirect longevity will be ignored and only results for direct longevity will be presented. Most of the correlations between breeding values of antibody levels with longevity had a negative sign (Table 6.2), indicating that a higher breeding value for antibody level is associated with lower longevity. However, only correlations of isotype IgA and IgM binding KLH were significantly deviating from zero ($p<0.05$) with estimates of -0.2082 and -0.2335 resp. Applying Calo's method revealed approximations of genetic correlations -0.3153 and -0.3397 resp.

Table 6.1: Means, standard deviation (std) and minimim and maximum of estimated breeding values for NAbs, for SCS of sample and for indirect (lvd) and direct (dld) longevity.

Trait	n	mean	std	min	max
EBV_klhigg1	97	-0.18	0.43	-1.62	1.42
EBV_klhiga	97	0.06	0.47	-1.02	1.20
EBV_klhigm	97	0.07	0.47	-0.93	1.14
EBV_lpsigg1	97	-0.10	0.42	-1.16	1.09
EBV_lpsiga	97	0.09	0.52	-0.87	1.63
EBV_lpgigm	97	0.07	0.46	-1.02	1.20
EBV_pgnigg1	97	0.06	0.20	-0.48	0.61
EBV_pgniga	97	0.13	0.45	-0.97	1.33
EBV_pgnigm	97	0.10	0.40	-1.09	1.28
EBV_ltaigg1	97	-0.09	0.16	-0.44	0.32
EBV_ltaiga	97	0.10	0.40	-0.86	0.92
EBV_ltaigm	97	0.02	0.29	-0.83	0.74
EBV_scs	97	-0.04	0.19	-0.57	0.37
EBV_lvd	97	56.85	233.53	-414.00	580.00
EBV_dld	97	59.04	232.92	-411.00	572.00

These results revealed that isotype IgA and IgM binding KLH are genetically correlated to longevity and therefore can be included into breeding for longevity. These measurements are taken in the first lactation and thus long before the actual culling of a cow is realized. Antibodies measured in first lactations has therefore good perspectives to serve as an early predictor in breeding for longevity. As a reference, other early predictors in the breeding value of longevity have genetic correlations of 0.20 - 0.30. Genetic correlation of SCS with longevity is higher (-0.407) although this might be based on repeated measurements of SCS (Weller and Ezra, 2015). Two further aspects that are related to the usefulness of incorporating antibodies into breeding are the relation to other early predictors and the possibilities that antibodies can be routinely measured in the milk samples. The latter is beneficial to improve reliability of breeding values.

Table 6.2: Correlations of breeding value for antibody level with breeding value for direct longevity (r_dld) and correlations after applying Calo method (r_dld_calo).

Trait	r_dld	r_dld_calo
EBV_klhigg1	0.0338	0.0602
EBV_klhiga	-0.2082	-0.3153
EBV_klhigm	-0.2335	-0.3397
EBV_lpsigg1	0.1085	0.1886
EBV_lpsiga	-0.1014	-0.1534
EBV_lpgigm	-0.0957	-0.1454
EBV_pgnigg1	0.0372	0.0851
EBV_pgniga	-0.0866	-0.1373
EBV_pgnigm	-0.1150	-0.1806
EBV_ltaigg1	-0.0649	-0.1491
EBV_ltaiga	-0.1092	-0.1687
EBV_ltaigm	-0.0629	-0.1149
EBV_scs	-0.1503	-0.2985

6.4 Genomic selection and future dairy cattle breeding

Genomic selection is used more and more and has become an important tool in dairy cattle breeding. Genomic selection, relies on the use of a large number of anonymous markers and typically does not make use of markers with a proven effect on the trait of interest. Genomic selection will increase selection response and the largest improvement is expected for traits that only can be measured late in life (Hayes et al., 2009). For a trait as longevity genomic selection is, therefore, very promising. The information on the longevity of a cow becomes available only after the cow is culled and thus no longer available for selection. There is quite some research on the detection of genes contributing to genetic variation of traits and on the function of these genes. Knowledge on which genes are responsible for antibody regulation and longevity can help to improve the accuracy of genomic prediction of longevity. Lopez et al. (2016) showed that the accuracy of genomic prediction can be improved by including markers with known effect. The improvement in accuracy was largest for traits with a low accuracy of genomic predictions. Including genes with known effect offers, therefore, an interesting option to improve genomic predictions of longevity.

In this thesis a genome wide association study (GWAS) on natural antibody was performed. GWAS methods has been successful in identifying associations between disease, or a trait of interest, and genes involved in regulating these traits, using markers evenly spread across the genome (Rosenberg et al., 2010). Genome-wide association studies provide insight into genes and biological pathways affecting the trait of interest, regardless of complications due to gene by environment and statistical interactions (Thomas, 2010).

The GWAS performed in this thesis (**Chapter 5**) revealed a total of 10 genetic markers significantly (FDR = 5%) or suggestively associated with natural antibody level. Regions on chromosome 21 (BTA21), were found to be significantly associated with natural antibody regulation, especially for isotype IgG. Within the significant regions several genes were located that are involved in the control and regulation of B cell activity and thus provide a link with the production of antibodies.

The genes TRAF3 and WDR20 are implicated in the control of immune cell signalling, relevant for B cells. It is noteworthy that TRAF3 is also involved in CD40 and BAFFR signalling, linking also TLR responses for instance against bacterial antigens such as PGN, LPS and LTA to the antibody levels measured against KLH.

The highlighted gene TP53BP1, which is controlling the isotype switch from IgM to IgG, in relation to KLH IgG levels is particularly relevant. Since also the immunoglobulin Heavy chain locus (IgH), located upstream of the highlighted region on BTA21, creates the prerequisite condition to be relevant for the observed association with KLH IgG levels.

This hints at the fact that part of the B cells that make IgM antibodies in first instance, apparently are class switched to IgG production. Implying that IgM NAb producing B cells are triggered by the immune system to respond and shift to IgG production. Whether that makes IgG binding KLH actually SpAb is unclear (and subject of a difficult discussion (which is not the topic of this thesis)). Anyway the fact that Ab to KLH are produced in significant quantities may be caused by a cross-reaction with an at present uncharacterized/unknown 'Self' antigen or alternatively by antigen of e.g. bacterial origin (e.g. Phosphatidyl Choline (PC)).

Another intriguing gene is PPP2R5C. Given the facts discussed in section 6.3.1 , this gene provides a link of the metabolic status of a cow with IgG NAb levels (and indirectly with IgM levels). PPP2R5C is involved in the control of carbohydrate and lipid homeostasis in the liver, this is relevant as homeostatis is likely affected when cows are suffering from negative energy balance. This is the first time a gene involved metabolic homeostasis is indicated in a GWAS, yet this seems relevant given the knowledge we have on difficulties cows have during lactation.

The results from the GWAS are interesting and trigger the imagination, however to be useful for implementation in genomic selection further studies are required. First the GWAS should be repeated with a larger group of animals from the same population (i.e. Canadian Friesian Holsteins). Alternatively the significant SNP should be tested in other populations to evaluate if the observations described in **Chapter 5** can be repeated. In that respect the "Weerbaar Vee" project would be a suitable option, since material has been stored in a biobank and phenotypes are already present.

Inclusion of the results from the GWAS study is expected to increase the accuracy of genomic predictions of natural antibody traits. Natural antibodies serve as an indicator trait for longevity. When including antibodies in genomic predictions of longevity, their characteristics of the different antibodies need to be taken into account. For example, natural antibodies binding KLH are expected to provide information on the number of natural antibodies and B1 type B-cells, and therefore the potential of a cow to induce an immune response to new intruders. The level of natural antibodies was found to be associated with longevity in sheep (Graham et al., 2010). Graham et al. (2010) found positive relations between auto-antibodies and longevity but negative relations between auto-antibodies and reproduction. Before introducing antibodies into selection decisions it is therefore important to consider the effects not only on longevity but also on other traits.

6.5 References

Abdel-Azim, G. A., A. E. Freeman, M. E. Kehrli, Jr., S. C. Kelm, J. L. Burton, A. L. Kuck, and S. Schnell. 2005. Genetic basis and risk factors for infectious and noninfectious diseases in US Holsteins. I. Estimation of genetic parameters for single diseases and general health. J. Dairy Sci. 88(3):1199-1207.

Balsari, A. and A. Caruso. 1997. Natural antibodies to IL-2. Biotherapy 10:25-28.

Banos, G., E. Wall, M. P. Coffey, A. Bagnall, S. Gillespie, G. C. Russell, and T. N. McNeilly. 2013. Identification of immune traits correlated with dairy cow health, reproduction and productivity. ploS one 8(6):e65766.

Baumgarth, N., O. C. Herman, G. C. Jager, L. E. Brown, L. A. Herzenberg and J. Chen. 2000. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. J. Exp. Med. 192(2):271-280.

Baumgarth, N., J. W. Tung and L. A. Herzenberg. 2005. Inherent specifities in natural antibodies: a key to immune defense against pathogen invasion. Springer semin Immun. 26:347-362.

Beaudeau, F., H. Seegers, C. Fourichon and P. Hortet. 1998. Association between milk somatic cell counts up to 400 000 cells/ml and clinical mastitis in French Holstein cows. Veterinary Record 143, 685±7.

Boes, M., A .P Prodeus, T. Schmidt, M. C. Carroll and J. Chen. 1998. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J. of Exp. Med. 188 (12): 2381-2386.

Bradley, A. J. 2002. Bovine mastitis: an evolving disease. The Vet. J. 164, 116-128.

Calo, L. L., R. E. McDowell, L. D. VanVleck and P. D. Miller. 1973. Genetic aspects of beef production among Holstein-Friesians pedigree selected for milk production. J. of Animal Sci. 37(3):676-682.

Carlèn, E., E. Strandberg and A. Roth. 2004. Genetic parameters for clinical mastitis, somatic cell score, and production in first three lactations of Swedisch Holstein cows. J. Dairy Sci. 87:3062-3070.

Cartwright, S. L., N. Begley, L. R. Schaeffer, E. B. Burnside, and B. A. Mallard. 2011. Antibody and cell-mediated immune responses and survival between Holstein and Norwegian Red x Holstein Canadian calves. J. Dairy Sci. 94(3):1576-1585.

Casali, P. and A. L. Notkins. 1989. CD5+ B Lymphocytes, polyreactive antibodies and the human B-cell repertoire. Immunol. Today 10 (11):364-368.

Cheng, H. M. and L. Chamley. 2008. Cryptic natural autoantibodies and co-potentiators. Autoimmunity reviews 7:431-434.

Ehrenstein, M. R. and C. A. Notley. 2010. The importance of natural IgM: scavenger, protector and regulator. Nature reviews Immunoloy, 10:778-786.

Graham, A. L., A. D. Hayward, K. A. Watt, J. G. Pilkington, J. M. Pemberton, D. H. Nussey. 2010. Fitness correlates of heritable variation in antibody responsiveness in a wild mammal. Science 330: 662-665.

Hayes, B., P., Bowman, A. Chamberlain and M. Goddard. 2009. Invited review: Genomic selection in dairy cattle: Progress and challenges. J. Dairy Sci. 92: 433-443.

Halasa, T., K. Huijps, O. Østerås, and H. Hogeveen. 2007. Economic effects of bovine mastitis and mastitis management: A review. Vet. Q. 29:18–31.

Harmon, R.J. 1994. Symposium: Mastitis and genetic evaluation for somatic cell count. Physiology of mastitis and factors affecting somatic cell counts. J. Dairy Sci. 77:2103-2112.

Hine, B. C., S. L. Cartwright, and B. A. Mallard. 2011. Effect of age and pregnancy status on adaptive immune responses of Canadian Holstein replacement heifers. J. Dairy Sci. 94(2):981-991.

Janeway, C. A., P. Travers, M. Walport, and M. Shlomchik. 2001. Immunobiology 5. 5 ed. Garland Publishing, New York.

Kohler, H., J. Bayry, A. Nicoletti and S.V. Kaveri. 2003. Natural autoantibodies as tools to predict the outcome of immune response? Scandinavian J. of Imunol. 58:285-289.

Lopez, M. S., H. Bovenhuis, M. van Son, Ø. Nordbø, E. H. Grindflek, E. F. Knol, J. W. M. Bastiaansen. 2016. Using markers with large effect in genetic predictions. In : Genomic selection for improved crossbred performance (PhD Thesis), p 85-105. Wageningen University, Wageningen, The netherlands ISBN :978-94-6257-631-5.

Lutz, H. U. ans S. Miescher. 2008. Natural antibodies in health and disease: An overview of the first international workshop on natural antibodies in health and disease. Autoimmunity reviews 7:405-409.

Lutz, H. U., C .J. Binder and S. Kaveri. 2009. Naturally occurring auto-antibodies in homeostasis and disease. Trends in Immunology 30(1): 43-51.

Machado, V. S., M. L. S. Bicalho, R. O. Gilbert, and R. C. Bicalho. 2014. Short communication: Relationship between natural antibodies and postpartum uterine health in dairy cows. J. Dairy Sci. 97:7674-7678.

Mallard, B. A., H. Atalla, S. Cartwright, B. C. Hine, B. Hussey, M. Paibomesai, K. A. Thompson-Crispi, L. Wagter-Lesperance. 2011. Genetic and epigenetic regulation of the bovine immune system: practical implications of the high immune response technology, p 53– 63. Proc. Natl. Mastitis Council 50th Annu. Meet. National Mastitis Council, Verona, WI.

Ochsenbein, A.F. and R.M. Zinkernagel. 2000. Natural antibodies and complement link innate and acquired immunity. Immunol. Today 21(12): 624-629.

Östensson, K. and Lun, S. 2008. Transfer of immunoglobulins through the mammary endothelium and epithelium and in the local lymph node of cows during the initial response after intramammary challenge with *E. coli* endotoxin. Acta Vet. Scand. 50(1), 26.

Parmentier, H. K., G. De Vries Reilingh, and A. Lammers. 2008. Decreased specific antibody responses to α-gal-conjugated antigen in animals with pre-existing high levels of natural antibodies binding α-gal-residues. Poultry Sci. 78:918-926.

Ploegaert, T. C. W., S. Wijga, E. Tijhaar, J. J. van der Poel, T. J. G. M. Lam, H. F. J. Savelkoul, H. K. Parmentier and J. A. M. van Arendonk. 2010b. Genetic

variation of natural antibodies in milk of Dutch Holstein-Friesian cows. J. Dairy Sci. 93: 5467-5473.

Ploegaert, T. C. W., B. J. Ducro, L. H. Oosterik, J. J. Van der Poel, T. J. G. M. Lam, H. K. Parmentier, J. A. M. Van Arendonk, H. F. J. Savelkoul, and E. Tijlhaar. 2010b. Relation of natural antibodies in milk of Holstein Friesian heifers with the risk for high somatic cell count and mastitis. In: Parameters for natural resistance in bovine milk (Phd Thesis), p 67-80. Wageningen University, Wageningen, The Netherlands. ISBN: 978-908585-827-0.

Pryce, J. E., R. J. Esslemont, R. Thompson, R. F. Veerkamp, M. A. Kossaibati, G. Simm. 1998. Estimation of genetic parameters using health, fertility and production data from a management recording system for dairy cattle. An Sci 66:3 577-584.

Rosenberg, N. A., L. Huang, E. M. Jewett, Z. A. Szpiech, I. Jankovic, and M. Boehnke. 2010. Genome-wide association studies in diverse populations. Nat. Rev. Genet. 11(5):356-366.

Rupp, R. and D. Boichard. 1999. Genetic parameters for clinical mastitis, somatic cell score, production, udder type traits and milking ease in first lactation Holsteins. J. Dairy Sci 82:2198-2204.

Rupp, R., Beaudeau, F. and Boichard, D. 2000. Relationship between milk somatic-cell counts in the first lactation and clinical mastitis occurrence in the second lactation of French Holstein cows. Preventive Veterinary Medicine 46, 99-111.

Rupp, R. and D. Boichard. 2003. Genetics of resistance to mastitis in dairy cattle. Vet. Res. 34:671-688.

Samoré, A. B., M. D. P. Schneider, F. Canavesi, A. Bagnate, and F. A. Groen. 2003. Relationship between somatic cell count and functional longevity assessed using survival analysis in Italian Holstein-Friesian cows. Livest Prod Sci. 80 (3):211-220.

Sharma, N., N. Singh, and M. Bhadwal. 2011. Relationship of somatic cell count and mastitis: an overview. Asian-Aust. J. Anim. Sci. 24:429-438.

Star, L., Frankena, K., Kemp, B., Nieuwland, M.G.B., Parmentier, H.K. 2007. Natural humoral immune competence and survival in layers. Poultry Sci. 86: 1090-1099.

Steeneveld, W., H. Hogeveen, H. W. Barkema, B. J. van den, and R. B. Huirne. 2008. The influence of cow factors on the incidence of clinical mastitis in dairy cows. J. Dairy Sci. 91(4):1391-1402.

Sun, Y., H. K. Parmentier, K. Frankena, J. J. van der Poel. 2011. Natural antibody isotypes as predictors of survival in laying hens. Poultry Science 90: 2263-2274, 2011.

Suriyasathaporn, W., Y. H. Schukken, M. Nielen, and A. Brand. 2000. Low somatic cell count: a risk factor for subsequent clinical mastitis in a dairy herd. J. Dairy Sci. 83(6):1248-1255.

Thomas, D. 2010. Gene--environment-wide association studies: emerging approaches. Nat. Rev. Genet. 11(4):259-272.

Thompson-Crispi, K. A., B. Hine, M. Quinton, F. Miglior and B. A. Mallard. 2012a. Short communication: Association of disease incidence and adaptive immune response in Holstein dairy cows. J. Dairy Sci. 95:3888-3893.

Thompson-Crispi, K. A., A. Sewalem, F. Miglior, and B. A. Mallard. 2012. Genetic parameters of adaptive immune response traits in Canadian Holsteins. J. of Dairy Sci. 95: 401-409.

Thompson-Crispi, K. A., F. Miglior and B. A. Mallard. 2013a. Incidence rates of clinical mastitis among Canadian Holsteins classified as high, average, or low immune responders. Clinical and Vaccine Immunol. 20:106-112.

Thompson-Crispi, K. A., F. Miglior, and B. A. Mallard. 2013b. Genetic parameters for natural antibodies and associations with specific antibody and mastitis in Canadian Holsteins. J. Dairy Sci. 96: 3965-3972.

Van Knegsel, A. T. M., G. De Vries Reilingh, S. Meulenberg, H. Van den Brand, J. Dijkstra, B. Kemp, H. K. Parmentier. 2007. Natural antibodies related to energy balance in early lactation dairy cows. J. Dairy Sci. 90, 5490–5498.

Van Knegsel, A. T. M., M. Hostens, G. de Vries Reilingh, A. Lammers, B. Kemp, G. Opsomer, and H. K. Parmentier. 2012. Natural antibodies related to metabolic and mammary health in dairy cows. Prev. Vet. Med. 103:287-297.

Wagter, L. C., B. A. Mallard, B. N. Wilkie, K. E. Leslie, P. J. Boettcher, and J. C. Dekkers. 2000. A quantitative approach to classifying Holstein cows based on antibody responsiveness and its relationship to peripartum mastitis occurrence. J. Dairy Sci. 83(3):488-498.

Weller, J. I. and E. Ezra. 2015. Environmental and genetic factors affecting cow survival of Israeli Holsteins. J. of Dairy Sci. 98:676-684.

Wijga, S., Bovenhuis, H., J. W. M. Bastiaansen, J. A. M. van Arendonk, T. C. W. Ploegaert. E. Tijhaar and J. J. van Poel. 2013. Genetic parameters for natural antibody isotype titers in milk of Dutch Holstein-Friesians. Anim. Genet. 44: 485-492.

Wilkie, B., and B. Mallard. 1999. Selection for high immune response: an alternative approach to animal health maintenance? Vet. Immunol. Immunopathol. 72(1-2):231-235.

Summary

Summary

The dairy sector has a big impact on food production for the growing world population and contributes substantially to the world economy. In order to produce food in a sustainable way, dairy cows need to be able to produce milk without problems and as long as possible. Therefore, breeding programs focuses on improvement of important traits for dairy cows. In order to improve desirable traits and obtain genetic gain there is a constant need for optimization of breeding programs and search for useful parameters to include within breeding programs. Over the last decades, breeding in dairy cattle mainly focused on production and fertility traits, with less emphasis on health traits. Health problems, however, can cause substantial economic losses to the dairy industry. The economic losses, together with the rising awareness of animal welfare, increased herd size, and less attention for individual animals, have led to an increased need to focus more on health traits. Longevity is strongly related to disease resistance, since a more healthy cow will live a longer productive life (longevity). The identification of biomarkers and the detection of genes controlling health and longevity, would not only greatly enhance the understanding of such traits but also offer the opportunity to improve breeding schemes. The objectives of this thesis therefore were 1) to find an easy measurable disease resistance related biomarker in dairy cows, 2) identify the relation between antibodies and longevity, 3) identify genomic regions that are involved in antibody production/expression. In this thesis antibodies are investigated as parameters for longevity. Antibodies might be a novel parameter that enables selection of cows with an improved ability to stay healthy and to remain productive over a longer period of time. In this thesis antibodies binding the naive antigen keyhole limpet hemocyanin (KLH) were assumed to be natural antibodies. Antibodies binding bacteria-derived antigens lipoteichoic acid (LTA), lipopolysaccharide (LPS) and peptidoglycan (PGN) were assumed to be specific antibodies. In **chapter 2** it was shown that levels of antibodies are heritable (up to $h^2 = 0.23$). Additionally, antibody levels measured in milk and blood are genetically highly correlated (± 0.80) for the two studied isotypes (IgG and IgM). On the other hand, phenotypically, natural antibodies (from both IgG and IgM isotype) measured in milk cannot be interpreted as the same trait (phenotypic correlation = ± 0.40). In **chapter 3 and 4** it was shown that levels of antibodies (both natural-and specific antibodies) showed a negative relation with longevity: first lactation cows with low IgM or IgG levels were found to have a longer productive life. When using estimated breeding values for longevity, only a significant relation was found between natural antibody level (IgM binding KLH)

and longevity. Lastly **chapter 5** reports on a genome-wide-association study (GWAS), to detect genes contributing to genetic variation in natural antibody level. For natural antibody isotype IgG, genomic regions with a significant association were found on chromosome 21 (BTA). These regions included genes involved in isotype class switching (from IgM to IgG). The gained knowledge on relations between antibodies and longevity and the gained insight on genes responsible for natural antibodies level make antibodies potential interesting biomarkers for longevity.

Samenvatting

De melkveesector speelt een belangrijke rol in het produceren van voedsel voor de groeiende wereldbevolking en heeft derhalve een substantiële bijdrage aan de wereld conomie. Om er voor te zorgen dat voedsel op een duurzame manier geproduceerd wordt, is het belangrijk dat melkkoeien zolang mogelijk melk kunnen produceren zonder veel problemen te ondervinden. Daarom zijn er fokprogramma's die focussen op het verbeteren van belangrijke eigenschappen van melkkoeien. Om gewenste eigenschappen van melkvee te verbeteren en om genetische vooruitgang te boeken, is er een voortdurende noodzaak om fokprogramma's te optimaliseren. Voor de optimalisering van fokprogramma's is het belangrijk dat er bruikbare biomarkers voor de gewenste eigenschappen gevonden worden. De afgelopen jaren is de melkveefokkerij voornamelijk geconcentreerd op productie- en vruchtbaarheidskenmerken en minder op gezondheid. Gezondheidsproblemen binnen de melkveesector leiden echter tot structurele economische verliezen. Deze economische verliezen tezamen met de verhoogde bewustwording van dierenwelzijn, een stijging van het aantal dieren per bedrijf en daardoor minder aandacht voor elk individueel dier, vormen aanleiding om meer aandacht te besteden aan gezondheidskenmerken in de melkveefokkerij. Levensduur van koeien is sterk gerelateerd aan ziekteresistentie, aangezien gezonde koeien ook een langer productief leven kunnen hebben. De identificatie van nieuwe biomarkers en het vinden van genen die verantwoordelijk zijn voor het aansturen van gezondheids- en levensduurkenmerken, zouden niet alleen meer inzicht geven in het begrijpen van deze eigenschappen, maar zouden ook kunnen bijdragen aan het verbeteren van fokprogramma's. De doelstellingen van het onderzoek in dit proefschrift waren daarom 1) het vinden van een makkelijk meetbare biomarkers voor levensduur in melkvee (antilichamen), 2) het identificeren van de relatie tussen niveaus van antilichamen en levensduur, 3) het identificeren van regio's in het genoom die verantwoordelijk zijn voor het aansturen van antilichaam egulatie. In dit proefschrift zijn antilichamen onderzocht als potentiële biomarkers voor levensduur. Antilichamen zijn mogelijk een bruikbare, nieuwe biomarker die gebruikt kan worden in fokprogramma's om te selecteren voor koeien met een verbeterd vermogen om gezond te blijven en daardoor ook een langer productief leven kunnen hebben. In dit proefschrift worden antilichamen die binden aan het naïeve antigen keyhole limpet hemocyanin (KLH) verondersteld als natuurlijke antilichamen. Antilichamen die binden aan antigenen afkomstig van bacterie-deeltjes zoals lipoteichoic acid (LTA), lipopolysaccharide (LPS) en peptidoglycan (PGN) worden verondersteld als specifieke antigenen. **Hoofdstuk 2** wordt getoond dat antilichaam niveaus erfelijk zijn, met een erfelijkheidsgraad tot 0.23. Daarnaast zijn antilichaam niveaus,

gemeten in bloed en melk genetisch sterk aan elkaar gerelateerd (± 0.80) voor de onderzochte isotypes (IgG en IgM). Daarentegen zijn de fenotypische correlaties die gevonden zijn tussen antilichaam niveaus in bloed en melk veel lager (± 0.40). Hierdoor kunnen antilichaam niveaus gemeten in bloed en melk niet gezien worden als dezelfde eigenschap. In **hoofdstuk 3 en 4** wordt aangetoond dat antilichaam niveaus (zowel natuurlijke als specifieke) een negatieve relatie hebben met levensduur: eerste lactatie koeien met lage IgM of IgG niveaus hebben een langer productief leven. In de analyse van fokwaardes voor levensduur werd alleen een negatieve relatie gevonden tussen levensduur en natuurlijke antilichamen (gebonden aan KLH) van het isotype IgM. In **hoofdstuk 5** wordt via een genome-wide-association studie (GWAS) aangetoond dat er genen zijn die verantwoordelijk zijn voor de variatie in antilichaam niveaus. Voor natuurlijke antilichamen van het isotype IgG werden significante associaties met betrokken regio's in het genoom gevonden op chromosoom 21 (BTA). Deze gevonden regio's bevatten genen die een rol spelen bij isotype-klasse verandering (van IgM naar IgG). De in dit proefschrift beschreven kennis over relaties tussen antilichamen en levensduur en het verbeterde inzicht in betrokken genen bij het reguleren van antilichaam niveaus, maken antilichamen een potentieel interessante biomarker voor levensduur.

Training and Supervision plan

WIAS Training and Supervision Plan

The Basic Package (3.0 ECTS[1])	
WIAS Introduction Course	2012
WIAS Course on Philosophy of Science and Ethics	2012
Scientific Exposure (12 ECTS)	
International Conferences	
64th EAAP Annual Meeting, Nantes, France	2013
10th WCGALP, Vancouver, Canada	2014
35th Society for Animal Genetics Conference, Salt Lake City, USA	2016
Seminars and Workshops	
F&G connection days, Vugt, 29 and 30 November	2012
WIAS Science Day, Wageningen, The Netherlands	2013
WPC Symposium: 'Healthy Food & Environment', Wageningen, The Netherlands, December	2013
WIAS Science Day, Wageningen, The Netherlands	2014
F&G Connection Days, Ellecom, The Netherlands	2014
WIAS Science Day, Wageningen, The Netherlands 2015	2015
WIAS Science Day, Wageningen, The Netherlands 2016	2016
Presentations	
Oral presentation at 64th EAAP, Nantes, France	2013
Oral Presentation WIAS Science Day 2015	2015
Poster presentation WIAS Science Day	2014
Poster Presentation at 10th WCGALP in Vancouver, Canada	2014
Oral Presentation 35th ISAG in Salt Lake City, USA	2016

In-depth Studies (8.6 ECTS)	
Indepth Immunology course Guelph, Canada	2015
Advanced Immunology Course UMCUtrecht	2015
Cursus Levensduur Melkvee, Wageningen Academy, the Netherlands	2015
Genetic Analysis using ASReml 4.0	2014
PhD students' discussion groups	
QDG: Quantitative Genetics Discussion Group	2012-2016
DOMO: Thursday Morning Meeting Immunology Discussion group	2013-2016
NDG: Natural Antibody and Animal Health Discussion Group	2014-2016
Statutory Courses (0 ECTS)	
Use of laboratory Animals, Wageningen, The Netherlands	2009
Professional Skills Support Courses (3 ECTS)	
Techniques for Writing and Presenting a Scientific Paper, Wageningen, The Netherlands	2015
Writing high impact science, Wageningen, The Netherlands	2015
Survival guide to peer review, Wageningen, The Netherlands	2015
PhD Career assessment	2015
Research Skills Training (3.5 ECTS) ASReml introduction Course, Wageningen, The Netherlands	2013
Introduction to R for statistical analysis, Wageningen, The Netherlands	2013
WHMIS (Workplace Hazardous materials	2015

Inventory System), University of Guelph, Canada	
Laboratory Safety, University of Guelph, Canada	2015
Animal Care Core Modules/ Ethics, University of Guelph, Canada	2015
Work with pigs as research animal, University of Guelph, Canada	2015
External training Period at University of Guelph, Guelph, Canada	2015

Didactic Skills Training (12 ECTS)

Group supervisor Inleiding Dierwetenschappen	2013
Practical Teacher, Applied Animal Biology (Cattle/Horses)	2013
Practical Teacher, Applied Animal Biology (Cattle/Horses)	2015
Supervising 1 minor MSc student Supervising 3 Bsc students	2015 2014-2015
Organizing practical for secondary school students 'Veebeoordelen', Wageningen, NL	2014-2015
Organizing practical for secondary school students 'Fokkerij & Genetica', Wageningen, NL	2015

Management Skills Training (5.5 ECTS)

Member of WIAS Associated PhD Students Council (WAPS)	2013-2014
Chair of the WIAS Associated PhD Students Council (WAPS)	2014-2015
Member of ABG Staff meetings Organisation	2013-2015
of 1st WPC Symposium 'Healthy Food & Environment', Wageningen, NL	2013

Education and Training Total	**47.6 ECTS**

Curriculum vitae

About the author

Britt de Klerk is born on the 2nd of July 1985 in Amsterdam. She was partly raised on a farm in Terschuur and graduated in 2004 from high school Alberdingk Thijm College in Hilversum. In the same year, she started her study Animal Science at Wageningen University, the Netherlands. She finished her Bachelor in 2008 by writing her bachelor thesis focussed on fertility in horses. In 2010 she completed her Master Animal Breeding and Genetics. During her Master she worked on 3 different theses; first a minor thesis at the Experimental Zoology group, entitled: Effect of swim training on bone and cartilage development in zebra fish. Secondly, she travelled to Rockhampton, Australia for an internship at CSIRO and work with Dr Haja Kadarmideen. In Australia she worked on a project to investigate worm- and tick resistance in different Australian beef cattle breeds, by using complex segregation analysis. Lastly, she worked on a major thesis focussing on genetic analyses of insect bite hypersensitivity in Dutch Gelder horses. During both her Bachelor and her Masters study Britt was working as student-assistent for the courses of Human & Animal Physiology I and II, were she assisted many different course practicals for bachelor students. After her graduation in 2010 Britt worked as research-assistent at the Animal Breeding and Genetics group in Wageningen, The Netherlands, where she worked on different research projects and was involved in assisting several bachelor- and master courses. In 2012 she started as PhD candidate on a project focussing on genetic analysis of antibodies and longevity in dairy cattle as part of the Dutch project “WeerbaarVee”. During her PhD project she was active as chair of the WAPS council (Wageningen PhD council of the Wageningen Institute of Animal Science graduate school (WIAS)). In 2015, she received a WIAS PhD fellowship to travel to the University of Guelph, Canada to perform research resulting in a scientific paper which is included in this thesis. In Canada she worked at the department of Pathobiology under the supervision of professor Bonnie Mallard. Final results of the total PhD project are included in this thesis entitled: : ”Antibodies and longevity of dairy cattle: genetic analysis”.

Over de auteur

Britt de Klerk is geboren op 2 Juli 1985, te Amsterdam. Ze is deels opgegroeid op een boerderij in Terschuur en heeft in 2004 haar VWO-diploma behaald aan het Alberdingk Thijm College In Hilversum. In dat zelfde jaar is zij begonnen met haar Bachelor studie Dierwetenschappen aan de Universiteit van Wageningen, in Nederland. Ze rondde haar Bachelor af in 2008 met het schrijven van haar Bachelor scriptie over vruchtbaarheid bij paarden. In 2008 startte Britt met haar Master met een specialisatie in Fokkerij en Genetica. In 2010 heeft zij haar Master afgerond. Tijdens haar master heeft Britt aan 3 verschillende scripties gewerkt; een minor scriptie op het gebied van Experimentele Zoölogie met als titel: “Effect van zwemtraining op bot- en kraakbeen ontwikkeling in zebravissen”, een stage in Australië bij CSIRO in Rockhampton, in samenwerking met Dr Haja Kadarmideen met als onderwerp genetisch onderzoek naar teken- en wormenresistentie bij verschillende vleeskoeien rassen met behulp van complex segregation analyses. En als laatste werkte zij aan een major thesis waarbij een genetische analyse is gedaan van staart- en manen eczeem bij Gelderse paarden. Gedurende haar Master- en Bachelor studie was Britt actief als student assistent bij het vak Mens en Dierkunde I en II waar zij verschillende practica begeleidde. Na haar Master is Britt werkzaam geweest als onderzoeks-assistent bij de leerstoelgroep Fokkerij en Genetica waar ze werkte aan verschillende projecten en betrokken was bij het assisteren van verschillende Bachelor- en Master vakken. In 2012 begon Britt aan haar PhD project over antilichamen en levensduur bij melkvee, onderdeel van het Nederlandse project “WeerbaarVee”. Tijdens haar PhD-periode is Britt actief geweest als voorzitter van de Wageningen PhD Council van de graduate school WIAS (WAPS). In 2015, ontving ze een WIAS PhD fellowship om een deel van haar PhD onderzoek in Canada uit te voeren, aan de universiteit van Guelph onder leiding van professor Bonnie Mallard. Het resultaat van het gehele PhD project is in dit proefschrift gepresenteerd onder de titel:” Antibodies and longevity of dairy cattle: genetic analysis”.

Publication List

Peer reviewed papers

de Klerk, B., B.J. Ducro, H.C.M. Heuven, I. den Uyl, J.A.M. van Arendonk, H.K. Parmentier, J.J. van der Poel. 2015. Phenotypic and genetic relationships of bovine natural antibodies binding keyhole limpet hemocyanin in plasma and milk. Journal of Dairy Science 2015 98:1-7.

van Altena, S.E.C., B. de Klerk, K.A. Hettinga, R.J.J. van Neerven, S. Boeren, H.F.J. Savelkoul, E.J. Tijhaar. 2016. A proteomics-based identification of putative biomarkers for disease in bovine milk. Veterinary Immunology and Immunopathology doi:10.1016/j.vetimm.2016.04.005.

Manuscripts in preparation

de Klerk, B., B.J. Ducro, H.C.M. Heuven, J.A.M. van Arendonk, H.K. Parmentier, J.J. van der Poel. 2016. Relation between antibody levels in milk and productive lifetime for Dutch dairy cows. *Submitted.*

de Klerk, B., B.J. Ducro, E. Mullaart, E. Koenen, J.A.M. van Arendonk, H.K. Parmentier, J.J. van der Poel. 2016. Relation between antibodies measured in milk and estimated breeding values for longevity of Dutch dairy cows. *Submitted.*

de Klerk, B., K. A. Thompson Crispi, M. Sargolzaei, B.J. Ducro, J.A.M. van Arendonk, J.J. van der Poel, B.A. Mallard. 2016. A genome-wide association study for natural antibodies measured in blood of Canadian Holstein cows. *In preparation.*

Santman-Berends I,, G. van Schaik, S. Carp-van Dijken, T. Lam, B. de Klerk, J. Keurentjes, I. den Uyl. Survival analyses based on natural antibody levels in dairy cows. 2016. *In preparation.*

Conference proceedings

de Klerk, B., B. Ducro, H. Heuven, I. den Uyl, J. van Arendonk, H. Parmentier, J. van der Poel. Natural Antibodies measured in Blood Plasma and Milk of Dutch Dairy Cattle. 64th European Association of Animal Production, Nantes, France, 2013.

de Klerk, B., B. Ducro, H. Heuven, I. den Uyl, J. van Arendonk, H. Parmentier, J. van der Poel. Can Natural Antibodies (NAbs) be used to select for more resistant dairy cows? WIAS Science Day, Wageningen, The Netherlands, 2014.

de Klerk, B., B. Ducro, H. Heuven, I. den Uyl, J. van Arendonk, H. Parmentier, J. van der Poel. Comparison of natural antibodies measured in milk and blood samples of Dutch Dairy Cattle. 10th World Congress of Genetics Applied to Livestock Production, Vancouver, Canada, 2014.

de Klerk, B., B. Ducro, H. Heuven, J. van Arendonk, H. Parmentier, J. van der Poel. Relationships between functional longevity of Dutch dairy cows and natural antibodies binding different antigens measured in milk. WIAS Science Day, Wageningen, The Netherlands, 2015

de Klerk, B., K. A. Thompson Crispi, M. Sargolzaei, B.J. Ducro, J.A.M. van Arendonk, J.J. van der Poel, B.A. Mallard. A genome-wide association study for natural antibodies measured in blood of Canadian Holstein cows. 35th Society for Animal Genetics Conference, Salt Lake City, USA, 2016.

Acknowledgements

Finally, the last part of my thesis, where I would like to use some space to say many thanks to all the people that helped me and stood by me during the process of my PhD project.

First of all I would like to thank my promotor Johan van Arendonk, who was always there for clarifying and fruitful discussions, followed by an update on Netherlands best soccer team: Ajax. Every time I had struggles or uncertainties, you were able to bring a lot of positivity into the project and into my head again. Johan, thanks for always be available and thanks for sharing all your good ideas and leading me into the right direction. You have been a great promoter and I really enjoyed the time we worked together.

Without my daily supervisors and co-promotors Jan, Bart and Henri I would not have been able to finish my thesis. It has been a great pleasure to work with all of you, and I already miss our fruitful (and long) discussions where we tried to understand the meaning of natural antibodies. Especially many thanks to Bart en Jan who have been there for me during my total project. Both of you were essential people for me, and I value all the time we worked and, sometimes even more important, laughed together. Bart and Jan, thank you for all the hard work and good times we had together and I hope we'll keep in touch.

Next, I would like to thank my colleagues from ABG, especially the ones that have been my roommates the past 4 years, and had to 'live' with me at least 40 hours a week; Gus, Mahlet, Hamed, Hadi, Sonia and Coralia. Additionally, I like to thank some other close colleagues from ABG who were always there for me during coffee-(cola-light-) breaks or for anything else, Bert, Kimberly, Rosilde, Piet, Dieuwertje, Anouk, Marcos, Andre, Ewa, Marzieh and Nancy. Thank you all guys for accepting me as I am ;) , for all the good conversations we had and for always being there for me whenever needed.

And last but not least, I would really like to thank and mention my appreciation for Ada and Lisette, you ladies are incredible and thanks for helping me with everything I needed! :) I can hardly imagine a life without you! You are the best!

I also want to express some extra appreciation to Henk Parmentier. You have been a great help with revising my papers, and are the best person to make my unclear stories a bit more clear! Thank you!

Many thanks to all members of the WeerbaarVee's project group, Ingrid den Uijl, Sanne Carpen-van Dijken, Christine van Altena, Henk Parmentier, Henri Heuven, Bart Ducro, Jan van der Poel, Erik Mullaart, Edwin Tijhaar, Judith Keurentjes and Inge Santman-Berendsen for good discussion material and for the positive closure of a successful WeerbaarVee project!

Many thanks to my friends that also happened to be my colleagues at Zodiac especially; Merel, Carla and Iris, for always being there for me when I needed another diet coke, or some chocolate, and to share PhD struggles or progress or to just talk about non-sense and silly horse stories.

Next I would like to thank the people I worked with in Guelph and that helped me with my last paper, Bonnie: Thank you very much for the great opportunity to work in your lab and learn more about the ins and outs of the immune system. And thanks for letting me ride your lovely horses! Also many thanks to the people in Guelph that helped me (stupid non-lab-experienced genetics girl) with all the lab work I performed, especially many thanks to Julie, Shannon and Leah who have been really patient with me! And Lauri and Neda, thank you for the language exchange series: 'dank je wel' and 'khasteh nabashi' :). And a lot of appreciation and thanks for Mehdi and Kathleen who helped me finishing my paper and where very helpful whenever I needed guidance. Especially many thanks to Emily for being my friend in Guelph for better and for worse ;) and thanks for being my lab-unicorn during the never ending-ELISA's we performed in the lab.

Off course I would like to thank my parents and sister. My parents for the brilliant gene-mixture, I turned out pretty okay ;) and for giving me all opportunities in life to develop myself. Special thanks to Mutti and Bo, for always being there for me, in darkness or in light, during the day or at night! You helped whenever I needed some advice, when I needed extra hands (thanks for being a blood-catcher-assistant Boe!) or just a shoulder to 'cry' on, and to make me smile whenever I stopped smiling for a while!

Also many thanks to my dear paranymphs, for not only being my paranymphs but also for being my best-buddies during my phd-ups-and-downs, for drinking, laughing, serious help, discussions and cheering me up during times I really needed it!

Of course I would like to thank my 'Taifoenies'!! You guys made me forget about the hard work sometimes and made me laugh with all your funny jokes! And even though 'time always continues ticking', we always make the best out of it! A very special friendship that means the world to me. Hopefully our Malaysian adventure will not be our last ;) AIJT!!

Thanks to all the people I have or haven't mentioned so far that helped me during my data-collection, in the lab or in the field: 'If you don't like pipetting, you should do it more often!' And 'Watch out for that bloody test-tube'.

Lastly, I would like to thank all my pets..... No kidding...! I wouldn't even go there!! Not enough space to mention all their names! ;) But they have been a valuable distraction during my relaxing moments!

To finalize, I can imagine I accidently forgot to mention people that have also been important for me during my PhD-project, and hopefully they know that I did not forget to mention them on purpose. Thanks to everyone! My PhD-project has been an amazing journey and it would not have been possible without all of you!

Colophon

The research presented in this thesis was part of a Dutch joint project WeerbaarVee, which is funded by the Dutch Ministry of Economic Affairs (The Hague, the Netherlands), Productschap Zuivel (Dutch Dairy Product Board, Zoetermeer, the Netherlands), CRV (Arnhem, the Netherlands), LTO Noord Fondsen (Zwolle, the Netherlands), Animal Health Services (Deventer, the Netherlands), and Wageningen University (Wageningen, the Netherlands)

The cover of this thesis was designed by Dave Haverkort.

This thesis was printed by Digiforce | Proefschriftmaken.nl, De Limiet 26, 4131 NC, Vianen, The Netherlands.